

Bigger isn't always better

The pharmaceutical industry's merger mania has done little to spur the innovation on which its future health will depend. Is it time to rethink the role of research within 'big pharma'?

In his heyday in the 1960s and 1970s, James Black demonstrated a Midas touch that turned base chemicals into pharmaceutical gold. An old-fashioned British pharmacologist who would think through his experiments for weeks before picking up a pipette, Black passionately believed in his duty to help cure disease — and in the capability of drug companies to help him do so. After leaving academia for ICI, he developed beta-blockers to treat hypertension. Later, working for Smith, Kline & French, his work on histamine H₂-receptor blockers revolutionized the treatment of stomach ulcers. Black's contribution to medicine was honoured with a Nobel Prize in 1988, and these two classes of drug are still bestsellers.

What wouldn't the world's pharmaceutical giants give for a return to the days when the steady arrival of new blockbuster drugs saw the industry riding high? Today it's a different story. The patents are running out on established earners and, despite the billions of dollars that the firms are pouring into research and development, the number of new drugs launched each year is falling. At the same time, the average cost of bringing a drug to market is rising, having doubled over the past 15 years; it now stands at as much as US\$800 million, according to the Tufts Center for the Study of Drug Development in Boston. Put these two trends together, and it is easy to see why analysts are warning that historical patterns of profit growth are unlikely to continue. Factor in the threat of a big earner being blown away by a successful patent challenge — as happened to GlaxoSmithKline's antibiotic Augmentin earlier this year — and things look bleak indeed.

There is no single explanation for what is going wrong. In part, the answer is that the 'easy' diseases have already been tackled with drugs that work well and are very cost-effective. A gastric ulcer, for instance, is caused by the oversecretion of stomach acids, mediated by unique biochemical pathways that are easy to hit selectively. Drug companies are now facing complex conditions with multiple causes, both environmental and genetic, such as asthma and Alzheimer's disease. And while the firms' publicity departments talk glibly about the prospect of 'personalized medicine', it's hard to find a pharmaceutical executive who has a clear idea of how to maintain profit margins in the pharmacogenomic era.

Getting together

Advances in both biological and chemical technologies are still yielding a multitude of drug targets and candidate compounds, but the pipeline from intriguing lead to finished product is leaky in the extreme. The vast majority of drugs — more than 99.9% — fall by the wayside in preclinical testing or in clinical trials. The latter failures are a particular problem, given the huge and rising cost of providing the clinical data to meet the stringent requirements of the US Food and Drug Administration and other regulators.

In recent years, the industry's answer to this pipeline problem has been an orgy of mergers. Pfizer, already the world's biggest-selling drugs company, last week unveiled plans to purchase Pharmacia for some \$60 billion in stock. GlaxoSmithKline, the product of a series of mergers including the mega-deal in 2000 between Glaxo Wellcome and SmithKline Beecham, is also reportedly also seeking another

partner. Put two giants with complementary late-stage pipelines together, cut costs by shedding staff with overlapping functions, add the mysterious ingredient of 'synergy', and everything is back on course — or at least, that's the theory.

The problem is that the merger medicine isn't working — as witnessed by the cool stockmarket reaction to these latest developments, and the steady decline in GlaxoSmithKline's share price since the 2000 amalgamation. Merged companies are still facing the same problems, such as how to work out when to fail a drug candidate to avoid throwing good money after bad. Worse, some industry insiders claim that the mergers are stifling innovation, rather than promoting it.

Speak to researchers at the bench and their immediate managers, and they will tell you that a merger is extremely disruptive. Projects are stalled and collaborations with start-up companies are put on ice until new research priorities are sorted out. Uncertainty reigns for months, if not years. And many feel that the resulting corporate hierarchies become too big and aggressive for a research group leader, or even a research director, to champion his or her projects successfully. It is hardly the sort of environment to entice an ambitious young researcher who wants to emulate Black's achievements.

New start

If disruption were temporary, the complaints could be put down to natural human discomfort with change. But the malaise seems deeper. Many of big pharma's best researchers are defecting for small biotech companies, where they soon feel revitalized and inspired. GlaxoSmithKline appeared to recognize the problem when it divided its research effort into six smaller drug-discovery centres modelled on biotech firms. But the recent departure of senior staff (see page 360) suggests that the experiment has not been an unqualified success.

If Black were entering his prime today, it seems highly likely that he would gravitate to an energetic start-up drug-discovery firm — even in the 1970s, his scientific individualism sat uncomfortably with the prevailing culture of the larger drug companies. So it's no surprise that, in search of the elixir of innovation, the drugs giants are increasingly turning to collaborations with start-ups (see *Nature* **414**, 482–483; 2001).

Many start-ups are engaging in highly speculative science that will never turn a profit, but others are quietly nurturing tomorrow's pharmaceutical money-spinners. Picking winners is notoriously difficult, which helps explain why big pharma is happy to let smaller partners take the risks. But perhaps it's time to start pushing this trend to its logical conclusion. The pharmaceutical industry's leaders could stop thinking about mergers, and instead turn over the early stages of drug discovery to nascent companies experimenting with new chemical, biological, genomic and computational technologies.

Big pharma's muscle will still be needed to push drugs through clinical trials and to supply them to global markets. But in future, its role in the early stages of the drugs pipeline might well be restricted to coordinating, facilitating and funding the work of the real innovators. If so, what we are currently witnessing may not be the start of the pharmaceutical industry's slow demise, but rather the first stirrings of its reorganization into a new and more viable form. ■





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Geneticist fears 'race-neutral' studies will fail ethnic groups

Peter Aldhous

The subject of race in America is a lightning rod that many researchers, especially geneticists, prefer to avoid. But a population

geneticist at Stanford University has grabbed it with both hands, claiming that 'colour-blind' approaches to medical genetics could actually discriminate against minorities.

The geneticist, Neil Risch, claims in a commentary article that political sensitivities may be leading some geneticists to abandon race as a variable in their studies — an approach that he thinks is dangerously misguided. "We need to value diversity, not to fear it," Risch and his colleagues argue in the online journal *Genome Biology* (see <http://genomebiology.com/2002/3/7/comment/2007>). "Ignoring our differences, even with the best of intentions, will ultimately lead to the disservice of those who are in the minority," they say.

Geneticists agree that a diverse population such as that of the United States cannot be considered as a homogeneous group. So, when trying to map the genes that confer susceptibility to particular diseases, or studying the performance of new drugs, it is useful to work out if the population can be divided into subgroups with particular genetic characteristics that might affect the results. One subgroup, for instance, might fail to respond to a drug that works well in the rest of the population.

Some geneticists are therefore studying genetic marker sequences to determine how people cluster into subgroups within the overall population. Last October, for instance, a team led by David Goldstein at University College London used some 40 markers to subdivide a genetically diverse sample of people from various populations throughout the world. They found that the resulting subgroups showed differences in the genes for enzymes that metabolize drugs (see J. F. Wilson *et al. Nature Genet.* **29**, 265–269; 2001). An accompanying editorial praised this "race-neutral" approach to population-genetic studies.

But Risch maintains that self-reported labels of racial origin — such as Caucasian, Asian, African American and Hispanic — can do the job just as well as sophisticated gene-clustering studies. "Effectively, you're just going to recreate those racial groups," he argues. What's more, Risch claims, if the data on genetic clustering are collected without regard to race, there is a tendency to assume that differences between the resulting subgroups are due to genetics — when they



In the mix: geneticists are at odds over whether to divide study populations along racial lines.

Biotech firms spurn university site

Rex Dalton, San Francisco

Faced with a continuing slump in US financial markets, biotechnology firms are turning down the chance to move into premises on a top university campus.

Nearly 100 researchers at the University of California, San Francisco (UCSF), are set to move into laboratories at the university's new Mission Bay campus in January.

But not a single technology firm has yet signed up to move into the new campus at UCSF, one of the United States' most prestigious biomedical research universities. Plans to construct a laboratory building for such firms are in limbo. An \$85-million commercial office sits vacant on the site, and construction of a second one has been halted.

UCSF officials say that they planned to accommodate professors in buildings adjacent to technology companies to develop "synergies", which they hoped would speed drug development as well as enhancing staff recruitment and retention.

But a weak economy, the cost of doing business in San Francisco and the fact that 20% of the city's offices are vacant seem to have dissuaded firms from moving to Mission Bay.

University officials are putting a brave face on the situation, professing optimism that high-technology tenants will eventually move in. But there is frustration that, after two years of marketing, prospective tenants keep dropping out.

"We are disappointed," says Bruce Spaulding, vice-chancellor for planning at UCSF. "But we remain optimistic — the long-range prognosis is good."

The project's troubles may alarm planners of other schemes in less auspicious locations, including Phoenix, Arizona, and Houston and Dallas, Texas, which have recently announced large investments aimed at attracting biotechnology businesses into close proximity to academic researchers (see *Nature* 417, 107; 2002).

might actually be due to socio-economic or cultural factors that just happen to correlate with race.

Risch also argues that minority groups should be selectively recruited into biomedical studies to ensure that they are sufficiently well represented to reveal any important differences from the population as a whole. Take a race-neutral approach, says Risch, and important information about minority health may be lost.

Not surprisingly, some scientists take issue with Risch's arguments. Harold Freeman, a prominent African-American researcher who is director of the National Cancer Institute's Center to Reduce Cancer Health Disparities, based in Rockville, Maryland, agrees that cultural factors should be considered in genetic studies. But he argues that racial labels are too crude to be useful. "Culture is not equal to race," says Freeman.

Worse, Freeman fears that the use of race as a variable in biomedical studies will perpetuate historical discriminatory attitudes. He adds that statistical findings from population-genetic studies may be wrongly applied to racial groups as a whole. If a drug is found to work less well in African Americans than in Caucasians, for example, it may end up being denied to individuals within the black population who might nevertheless benefit from it.

Goldstein worries that the tone of the Risch paper will bring unwanted political overtones into what ought to be a technical discussion. He accepts that it can be useful to divide populations into subgroups that reflect the geographical origins of their members' ancestors. However, Goldstein says that "race is not a terribly good framework. I believe we can do better. This is a technical question. I think that Neil has politicized it too much."

Risch argues that his article is merely responding to statements made in the scientific literature by other commentators. For instance, an editorial in *The New England Journal of Medicine* last year argued that "race is biologically meaningless" (see R. S. Schwartz *N. Engl. J. Med.* **344**, 1392–1393; 2001).

Francis Collins, director of the National Human Genome Research Institute in Bethesda, Maryland, feels that Risch makes some valid points. He points out that the international 'haplotype map' project, an effort to discover disease-susceptibility genes (see *Nature* **412**, 105; 2001), includes DNA samples from the main geographical areas of the world. But Collins thinks that Risch was unwise to frame the issue in terms of racial labels. "It's open to broad misinterpretation," he says. "I'd be happier if we could get away from the highly charged terminology of race and refer instead to geographical origin of ancestors."

Bioethics council demands tighter rules on gene patents

David Adam, London

Patents on DNA sequences are granted too easily, too often and with too little scrutiny, says an investigation into gene patenting by Britain's foremost bioethics body.

Thousands of recent patents asserting rights over DNA sequences are of "doubtful validity", according to a report released on 23 July by the Nuffield Council on Bioethics. The report says that, in future, patents involving DNA sequences should be issued as an exception, not as a rule.

Sandy Thomas, director of the council — which is operated and funded by the UK government's Medical Research Council and two London-based charities, the Wellcome Trust and the Nuffield Foundation — says the assessment is the broadest attempt yet to take stock of gene patenting after what she terms the "gold rush" that followed the publication of the human genome sequence last year.

"We are concerned that, for patents involving DNA, the patent system is in danger of not achieving its main goal: to stimulate innovation for the public good," Thomas says. Technology that allows computer users to identify genes and then patent them by merely sifting published sequences makes a mockery of the idea that applicants should demonstrate "inventiveness", she argues.

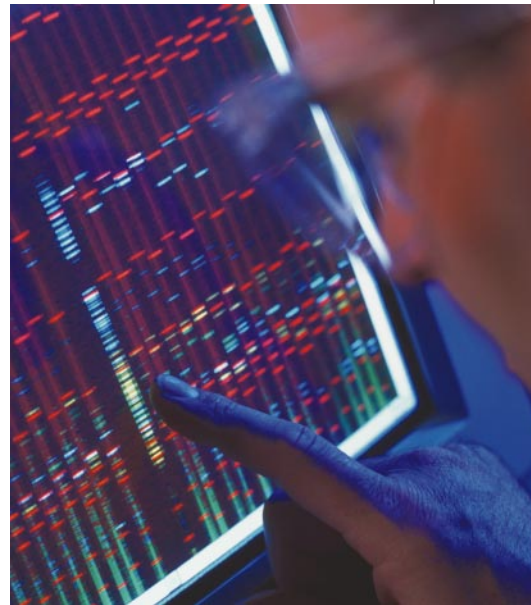
Previously, researchers had to identify, isolate and clone a gene before deciphering its sequence. "We're talking about a reassessment of the system," Thomas says, "not just a little bit of tightening here and there."

The report's authors hope their work will help to inform the decision-making processes of patent offices around the world. Some fear that existing patent regimes are too heavily influenced by pressure from patent applicants and their employers, and are not responsive enough to the interests of other researchers, who want open access to gene sequences, or of society as a whole.

To obtain a patent, applicants must convince patent offices that their innovation is not only novel and inventive, but also useful. The Nuffield report argues that, in many cases, the rigorous application of these existing criteria would reduce the number of gene patents issued, without any new rules.

The world's three largest patent systems, in Japan, Europe and the United States, currently treat each criterion differently. The US patent office is generally the most relaxed in issuing patents that involve DNA sequences, although it has tightened its scrutiny of utility in response to criticism that it was awarding patents on sequences with unproven or purely speculative uses (see *Nature* **403**, 3; 2000).

Rainer Moufang, a patent lawyer with the



Market speculation: has the availability of the human gene sequence led to a gold rush?

European Patent Office in Munich, says that European regulations have also been tightened and that applicants must show a commercial application before a patent is granted. The report's conclusion that patents on DNA should be the exception rather than the rule is "rather a difficult prognosis", says Moufang. "For us to change our practice we would need a clear hint from legislators," he adds.

Applications for patents on gene sequences that could be used to diagnose diseases should receive particular scrutiny, the report says. It contends, for example, that patents held by the US company Myriad Genetics on the *BRCA1* gene, which is linked to susceptibility to breast cancer, give the company effective control over the sequence, stopping others from developing alternative diagnostic tests. Some of Myriad's European patents are being challenged by French researchers (see *Nature* **413**, 95; 2001).

Deryck Beylleveld, director of the Sheffield Institute of Biotechnological Law and Ethics at Sheffield University, UK, welcomes the report's suggestions. "They are workable in both principle and practice," he says. "The obstacles will appear when they run into the politics."

Political leaders associate patents with competitiveness, says Beylleveld, and will be reluctant to discourage gene patenting. "We no longer have a patent system that rewards inventors for their creativity," he claims, "but one that essentially rewards investors for their investment."

TEK IMAGE/SPL

Biologists angered by database access fee

Alison Abbott

Geneticists are up in arms over the introduction of a subscription fee for access to the protein databases owned and maintained by Incyte Genomics, a private company based in Palo Alto, California.

The databases, which contain information about protein structure and function in important model organisms studied by biologists, had been free to academics. But Incyte last month introduced an annual access charge of \$2,000 per laboratory — a move that has already prompted an effort to secure more funding for public databases.

Many biologists claim that they would be prepared to pay a small fee to support the maintenance of high-quality databases — even though most of the data were generated by the researchers themselves in publicly funded labs. But some are crying foul over the size of Incyte's fee. "The fee is simply too high for small laboratories," says Gustav Ammerer, a yeast geneticist at the University of Vienna.

Incyte's database collection, known as the Proteome BioKnowledge Library, covers model organisms such as yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) and the nematode *Caenorhabditis elegans*, and also features some mammalian and microbial databases.

The library was founded in 1995 by James Garrels, whose company, Massachusetts-based Proteome, offered academics free access to the data but charged industrial users. Garrels sold the company to Incyte at the end of 2000, when he assured geneticists that the databases would remain free on the web to academics. Incyte was unavailable for comment on the reasons for the change.

The move has already caused researchers some difficulties. Peer Bork, a bioinformaticist at the European Molecular Biology Laboratory in Heidelberg, had to withdraw some information from a recent paper (C. von Mering *et al. Nature* **417**, 399–403; 2002) just before it was published, when Incyte changed the terms under which the data in it could be shared. The paper compared different analyses of protein interactions in yeast.

But with the introduction of the access fee, restrictions have become even tighter. "Now I don't know how to respond to enquiries about the data we used," Bork says, adding that he is unsure whether he can share his data with other researchers.

Bork and other researchers say that Incyte is just filling a void caused by a lack of funding for public-sector databases. Annotation — the process of assigning functional and other information to the genes and proteins in databases — is a skill that needs to be paid for, Bork says. "If a company is prepared to fill in the niche, then the argument is only with the pricing policy," he says.



Peer Bork (left) and Michael Cherry would like to see more money for public protein databases.

Peter Okkema, a biologist at the University of Illinois at Chicago, agrees that the controversy over commercial databases "is a matter of price rather than principle". When he learned in May that a subscription fee for Incyte's databases was imminent, he sent a letter signed by 160 scientists to Incyte's board of directors requesting reconsidera-

tion, but received no reply, he says. The silence, he adds, reveals a further problem with Incyte — its alleged lack of communication with the scientific community.

Chris Hogue, who runs BIND, a protein-interaction database based at the Samuel Lunenfeld Research Institute in Toronto, says that active input from researchers often isn't enough to keep a database going. Researchers "usually prefer someone else" to do the work needed to keep the database in shape. "My plea to researchers is to take database curation into your own hands," he says. "Everyone has to pitch in if they are to be freely available."

In the United States, scientists running model-organism databases in the public sector are lobbying for more support from the National Institutes of Health. Michael Cherry of Stanford University, who helps to run the *Saccharomyces* Genome Database there, says that more funds for its expansion would help to compensate those who cannot afford to access Incyte's yeast database. ■

Japan gives scientists political role

David Cyranoski, Tokyo

The scandal that has surrounded the discovery of bovine spongiform encephalopathy (BSE) in Japanese cattle has prompted the country's government to hand over a rare degree of responsibility to scientists. As of next year, ministers will follow the recommendations of a newly created committee of researchers when it comes to food-safety issues.

"It's an unprecedented amount of authority for scientists," says Takashi Onodera, an immunologist at the University of Tokyo. "The committee members will be like government officials."

Japan's first case of BSE was confirmed last September. The likely cause was contaminated meat and bone-meal that was imported from Britain and used as cattle feed. But a scandal erupted when the government was criticized for allowing such feed to be imported until 1996, despite it being banned in Europe in 1990. Four cases of BSE have now been confirmed.

This April, an independent report commissioned by the agriculture ministry found that the government had failed to take the opinions of food-safety specialists into account, and that the close relationship between politicians, bureaucrats and the beef industry had prevented it from responding appropriately to the threat of BSE.

The new committee will consist of five or six specialists in fields such as microbiology and food additives. It will submit its reports to the minister in charge of public safety.



Once bitten: agriculture minister Tsutomu Takebe will get scientific advice on food issues.

These reports are expected to have significant binding power. "If I don't follow the committee's recommendations, I'll get fired," says agriculture minister Tsutomu Takebe.

But some researchers are worried that the government will fill the committee with scientists who will tell ministers whatever they want to hear. "Judging from the government's past record, they will probably avoid anyone who would raise severe criticism," says Masanori Fukushima, an epidemiologist at Kyoto University.

Pleas to include a representative of a consumer group on the committee have already been rejected. "We want to keep the committee scientific," an agriculture ministry official explains. ■

Lords blast red tape in animal experiments

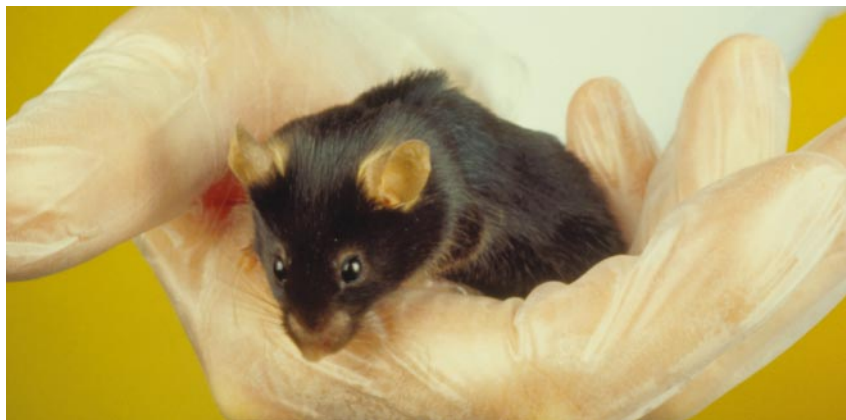
Natasha McDowell, London

Britain's system of regulating animal experiments — one of the toughest in the world — has become far too bureaucratic, says a report due to be released by a House of Lords select committee this week.

The Lords charge that current government funds for researching alternatives to animal experimentation are “meagre”, saying that more should be done to promote such methods.

Calling animals “highly imperfect models”, but acknowledging the necessity of their continued use in research, the committee also recommends that the public should get more information about what animal experiments are done, and why.

The report, which was written by the Select Committee on Animals in Scientific Procedures, advocates reducing the length of the forms needed to acquire a typical licence for animal experimentation from a minimum of 40 pages down to about 10 pages.



A stronger search for alternatives to animal experimentation is being urged by a House of Lords study.

“There is too much bureaucracy, which hampers scientific research and can even harm animal welfare,” says Lord Smith of Clifton, a professor of politics and the committee's chairman. He adds that the Home Office's

annual budget of £280,000 (US\$438,000) to promote the reduction, refinement and replacement of animal experiments is “hopelessly inadequate”. The committee calls for more money and a new national centre to serve as a focus for this work.

It is not unusual for select committee reports to gather dust in London after publication, but researchers are optimistic that this latest document will generate a response from the government — especially given the depth of the inquiry that preceded it, the government's strong public support for science, and the trouble that scientists are currently experiencing with animal regulations.

Biologists welcomed the committee's call for less arduous regulation. “It is right that the public has assurance of high standards,” says Richard Morris of the University of Edinburgh, who uses transgenic mice to develop models of Alzheimer's disease, “but scientists doubt the necessity for regulation to be accompanied by such cumbersome bureaucracy.”

Morris says that because of the regulations it is currently “almost impossible” for biomedical researchers in Britain to collaborate with foreign scientists “without flying them in months ahead to sit examinations and secure licences”.

But Gill Langley, a biologist who works as a scientific adviser to the Dr Hadwen Trust for Humane Research in Hitchin, north of London, says that the Lords should have focused more strongly on the need to find alternatives to animal experiments. She says that the committee should have proposed a centre dedicated entirely to replacing animals, akin to the European Centre for the Validation of Alternative Methods in Ispra, Italy.

The report also observes that the inclusion of all transgenic animals in government statistics on animal experiments — whether they are actually being experimented on or not — is artificially inflating these statistics.

US labs bemoan lack of stem cells

Kendall Powell, Washington

A year after President Bush publicly wrestled with how best to regulate the use of human embryonic stem cells in research, the availability of approved cell lines remains tightly constrained, US biologists say.

Wendy Baldwin, deputy director of extramural research at the National Institutes of Health (NIH), told the President's Council on Bioethics on 11 July that “in excess of two dozen” such cell lines are characterized and available for research. But this number does not tally with what researchers actually have to hand. Most contacted by *Nature* put the figure closer to five or six.

Last August, Bush restricted research to cell lines already in existence (see *Nature* 412, 665; 2001). Later that month, the government said that the NIH had identified

64 eligible cell lines and would establish a registry to aid their distribution to researchers. The registry now lists 78 cell lines from 14 sources in 6 countries.

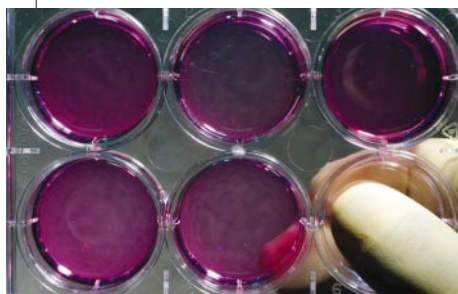
But most of the lines have not been characterized or tested for long-term growth in the lab. Others are duplicate lines or require company-approved collaboration for access. “There is no lab that works with more than half a dozen of these lines,” says Ali Brivanlou, an embryologist at Rockefeller University in New York.

NIH molecular biologist Ron McKay, whose work with mouse embryonic stem cells has produced functional transplanted neurons (J.-H. Kim *et al.* *Nature* 418, 50–56; 2002), also estimates that only about five cell lines are ready for use by US researchers. Far more cell lines will be needed to pursue therapies of the future, biologists contend.

Researchers face several hurdles in obtaining lines that the NIH regards as available — import permits are often required, fees of about \$5,000 per line must be paid, and, most awkwardly, deals have to be struck on intellectual property rights over any derived invention.

In a bid to speed up the flow of cells into the laboratory, the NIH has distributed \$3.5 million in grants to companies that hold the lines to fund their distribution and characterization, as well as to provide relevant training for researchers.

♦ <http://escr.nih.gov>



US researchers can get rodent embryonic stem cells (above), but human cells are more elusive.

GETTY IMAGES

J.C. REVY/SPL

Futurists predict body swaps for planet hops

Geoff Brumfiel, Washington

Direct brain-to-brain communication and the transfer of minds between bodies seem more like the stuff of Hollywood movies than of government reports — but these are among the advances forecast in a recent report by the US National Science Foundation and Department of Commerce.

“Improving human performance has been a dream for centuries,” says Mihail Roco, chairman of the government-funded National Nanotechnology Initiative, and lead author of the study. Dreams of herculean strength and everlasting life are currently constrained by the limitations of the human body. But the report — *Converging Technologies for Improving Human Performance*, released on 8 July — says that the convergence of nanotechnology, biotechnology, computer science and cognitive science may help to break those limits in the next 20 years.

Combining these fields will allow human technology to be seamlessly integrated with biological systems, say the authors, who come from a mixture of academic and industrial backgrounds. They suggest it might be possible to use nanorobots to repair damaged or defective body parts, or to improve peoples’ brains so that our memories never fade.

The report doesn’t stop there. If technology



and biological systems could be integrated absolutely, the authors claim it might be possible to ‘upload’ your mind from your body and transmit it to another world, such as Mars. Once there, you could download your mind into a different body and experience the planet. And by periodically uploading your mind to new bodies, it suggests, you could live forever.

Other futurist furrows ploughed in the report include the idea of individuals communicating using brain implants, and computer models that could predict how societies behave and evolve. The authors propose that

such models could be used to eliminate trends such as fundamentalism or fascism.

Presidential science adviser John M. Marburger acknowledges that some of ideas may seem a little extreme, but he believes they cannot be ignored. “Topics that seemed far out even a few years ago are much closer than we anticipated,” he says. “It would be irresponsible for us to dismiss these ideas.” Rita Colwell, director of the National Science Foundation, agrees. “It’s just a lovely report,” she says. “It provides an awfully good justification for the investments we’re making now.”

But not everyone is convinced. “I can’t imagine what they plan to do with the damn thing,” says Bob Park, director of public information at the American Physical Society. He believes that the ideas are too far-out to have much of an impact on current research funding. “You can’t make plans for technology so many years in advance,” says Park.

Others say that social behaviour is too complex to be reduced to a set of discrete variables. Modelling can help social scientists to understand trends, says Don Brenneis, president of the American Anthropological Association and a researcher at the University of California, Santa Cruz, but it won’t allow them to engineer ways out of social problems.

♦ <http://wtec.org/ConvergingTechnologies>

Imbalance a sticky issue among stamps of distinction

David Adam, London

As the acknowledged leader in your research field, you’ve earned the respect of colleagues, and that phone call from the Nobel prize committee will surely not be long in coming. But have you got what it takes to appear on a postage stamp? Very possibly, if you’re a physicist in Germany, according to a new survey — but definitely not if you’re a British mathematician.

Bob Jones, a retired chemist and keen stamp collector living in Meols in north-west England, scoured the entire philatelic output of Britain, France and Germany between 1951 and 1990 for sticky-backed science images.

“In an age where we no longer erect statues to our heroes, being celebrated on a postage stamp is an important mark of distinction,” Jones says. “But most specialists think their own area is not properly represented.”

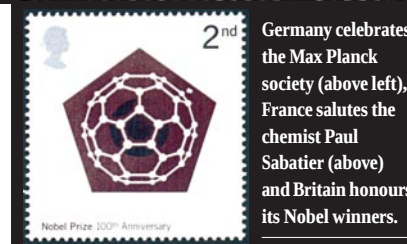
Jones’s trawl through national catalogues for the period discovered scientific images on 63 (6.2%) of the 1,022 commemorative stamps issued in Britain. In France it was 85 (4.7%) from 1,788 stamps, whereas science



appeared on 49 (3.6%) of the 1,365 stamps produced by the then West Germany.

Where science does appear, countries differ in their approaches. More than 80% of French science stamps in the survey celebrate individuals; in Germany the proportion is nearer two-thirds, whereas a mere one British science stamp in ten carries a portrait. This is perhaps in deference to Queen Elizabeth II, whose silhouette adorns every British stamp, encouraging designers to illustrate concepts, rather than people, in their work.

There are also different national partialities for particular disciplines. Biological science, including medicine, leads the way in the United Kingdom (on 33 stamps) and France (28 stamps), but appears



Germany celebrates the Max Planck society (above left), France salutes the chemist Paul Sabatier (above) and Britain honours its Nobel winners.

less often on German stamps (10) than do physics (33) and maths (12). Although British chemists have claimed 36 Nobel prizes, the subject has appeared only 13 times on the country’s stamps. And UK mathematics or mathematicians haven’t appeared once.

Unanimous vote puts Venus mission back on track

Paris The European Space Agency's (ESA's) mission to Venus is set to make an unexpected comeback.

Venus Express, originally slated for a 2005 launch, is designed to use the technology and industrial teams already in place for ESA's 2003 Mars Express mission. But David Southwood, head of the agency's science programme, dropped the project this May because of fears that the instruments for the mission would not be ready in time (see *Nature* **417**, 474; 2002).

The decision provoked a strong response from scientists, who see the chance to study the hot and corrosive atmosphere of Venus as having significant scientific potential. At a meeting on 11 July, ESA's Science Programme Committee unanimously voted in favour of retaining Venus Express. A final decision will be made in early November, when it will be known whether Italy, the only country not yet able to commit its payload, can do so in time to meet the mission deadline. Preparatory work on the mission will begin in the meantime.

Drug-discovery centres lose another director

London Bad news is flowing thick and fast for the British pharmaceutical company GlaxoSmithKline (GSK).

Frank Walsh, director of the company's neurology drug-discovery centre in Harlow, Essex, resigned earlier this month for personal reasons. He follows Martin Rosenberg, director of GSK's antibiotic drug-discovery centre in Upper Providence, Pennsylvania, who retired in May 2001.

GlaxoSmithKline's six drug-discovery centres, created last year, are modelled on small biotechnology businesses in order to spur competition between groups and reduce the time taken to bring drugs to market (see *Nature* **410**, 5; 2001).

The departure comes as GSK is mounting legal challenges to retain patent protection of Augmentin, the company's blockbuster antibiotic and, with US\$2 billion of annual sales, its biggest earner. GSK shares have lost almost half their value over the past year.

Supercomputer network gets wired up

Chicago The first connections in what will be the world's most powerful scientific computing network were switched on earlier this month.

The I-WIRE (Illinois Wired/Wireless Infrastructure for Research and Education) network, which links three Illinois

supercomputing facilities, is the first part of the US\$53-million TeraGrid project to go live. The largest of several similar projects under development around the world, it will allow users anywhere to access supercomputing and advanced visualization systems from their desktops.

Once complete, TeraGrid will offer computing power in the region of 16 teraflops (trillions of calculations per second) and access to visualization software capable of handling 1,000 terabytes of data. Researchers are now developing the 'middleware' needed to manage the system, and expect the first scientific use to begin later this year.

♦ www.teragrid.org

Space engineers search for missing re-entry vehicle

Moscow A spacecraft carrying an innovative re-entry system has gone missing somewhere on the sparsely populated Kamchatka peninsula in northeastern Russia.

The multi-layer inflatable heat shields of the Inflatable Re-entry and Descent Technology (IRDT) system — a joint venture between the European Space Agency and the International Science and Technology Centre in Moscow — act as parachutes to ensure a safe landing, as well as protecting the spacecraft from the heat generated during re-entry into the Earth's atmosphere. Engineers say the technology could be used to return small payloads from the International Space Station, independently of the Space Shuttle.

Initial tests in February 2000 were successful. But during a second test on 12 July, the shuttlecock-shaped craft had to cope with speeds of 7 kilometres per second, similar to those experienced during re-entry. Engineers lost contact with the craft after it separated from its launch rocket, and were still searching for it as *Nature* went to press.



Spaced out: a craft carrying heat shields (shown) was lost during a test flight over Russia.

ESA/NPO LAVOCHKIN

Bug power comes under the spotlight

Washington The potential of microbes to tackle problems of energy production and environmental clean-up is to be explored under a new US\$103-million programme funded by the US Department of Energy.

The “Genomes to Life” project will unravel the regulatory gene networks and protein complexes found in microbes that are involved in the atmosphere and ocean carbon cycles, and those that could potentially clean up sites contaminated with metals and radionuclides. Microbes that might be used to produce hydrogen for powering electricity-producing fuel cells will also be studied.

The funding, announced by the department on 23 July, will cover five different projects for five years.

♦ <http://doegenomestolife.org>

Lab head retires to seek a better balance



Lura Powell described the pace of her job as ‘gruelling’.

San Diego Lura Powell, the only woman to head a major US Department of Energy laboratory, has announced plans to retire after just two years in the job.

Powell, director of the Pacific Northwest National Laboratory in Richland, Washington, said on 19 July that she will step down at the end of the year. An analytical chemist, Powell

cited the gruelling pace of travel and job requirements. “It got to the point where I needed more balance in my life,” she said.

The laboratory carries out research on the environment, energy, health sciences and national security.

♦ www.pnl.gov

Shot in the arm for Norwegian research

Oslo Plans to push Norway up the league table of international science have been unveiled by the Norwegian Research Council.

A 1998 government report on the country’s research effort, which highlighted Norwegian researchers’ poor citation index ratings, led to last month’s

announcement that 13 centres of excellence in basic research are to be created. The government will provide 19 million euros (US\$19 million) annually over ten years to set up the centres, which will cover subjects such as mathematics, neuroscience, petroleum research, aquaculture and climate change. Other public and private bodies will cover running costs.

The 13 centres, to be based in universities and institutes across Norway, were chosen after a competitive call for proposals. A panel of international experts evaluated the short-listed projects on scientific merit and other criteria, including their potential to attract international researchers and the scientific quality of the host institution.

Compensation pay lightens the load for fired chemist

San Diego Anthony Czarnik, a combinatorial chemist and former chief scientific officer of San Diego biotechnology firm Illumina, was awarded US\$7.2 million on 16 July after a jury ruled that he was maliciously fired because of a medical condition.

Czarnik, who co-founded Illumina, brought the case after he left the company in September 2000. His lawsuit says that executives forced him out after learning that he suffered from depression, for which he took medication, and because he complained about potential data misrepresentations to possible investors.

Officials at Illumina, which develops high-throughput genetic-analysis technology, say they will appeal against the verdict. Czarnik now works at Sensors for Medicine and Science in Germantown, Maryland.

Pop star aims to follow Major Tom into space

Washington In what may rate as one of the most ambitious publicity stunts of all time, Lance Bass, a singer with the successful boy band *NSYNC, is attempting to travel to the International Space Station (ISS).

Bass visited Russian spaceflight training facilities this month in Star City, close to Moscow. Rosaviakosmos, the Russian aviation and space agency, confirmed last week that it was considering allowing the pop star to join a flight to the ISS. Bass would take a seat on a Soyuz spacecraft that is due to visit the station in October.

Russia has sent two space tourists to the ISS in the past year, reportedly receiving around US\$20 million on each occasion.

All living things, online

Can taxonomy shed its dusty image and reinvent itself as a vibrant discipline for the Internet age? Virginia Gewin talks to the pioneers who are trying to turn this vision into reality.

Taxonomy has an image problem. Perennially short of funding, its grey-ing practitioners are seen as dry and dusty — just like the museums and herbaria in which many of them work. “People who do taxonomy are depicted as postage-stamp collectors,” says Patrick Kociolek, executive director of the California Academy of Sciences in San Francisco, and an authority on the taxonomy of diatoms — algae that secrete cell walls made from glass.

Repeated reports from concerned organizations have echoed Kociolek's observation, describing a discipline in seemingly inexorable decline. But hope may be at hand, in the form of initiatives that aim to reinvent taxonomy for the online generation. One project, called the Catalogue of Life, is taking firm steps to establish a federation of interoperable databases documenting the world's taxonomic knowledge. Another group, the All Species Foundation, is even talking about naming and describing all living species within a single human generation.

That would be an incredible feat. There is currently no consensus on the total number of species: estimates range from 4 million to 100 million. In the 250 years since the great Swedish naturalist Linnaeus invented the modern taxonomic framework, roughly

Web of life: initiatives such as the All Species Foundation and Species 2000 aim to haul taxonomic information out of museums and herbaria to make it more readily accessible on the Internet.

hopes to quadruple the number of species described each year — a figure that currently stands at about 15,000. Today, there are at most 10,000 taxonomists worldwide, few of whom are in the developing countries that contain most of the Earth's biodiversity. All Species aims to double the taxonomic expertise in these countries by recruiting 'parataxonomists' trained to collect, sort and document specimens.

Everything counts

Janzen helped to pioneer this concept through his involvement with the first all-taxa biodiversity inventory (ATBI) at the Guanacaste Conservation Area in Costa Rica. An ATBI is an audit of the entire biodiversity within up to 100,000 hectares of an ecosystem. Only three have ever been started — the others are in Hawaii and the Great Smoky Mountains National Park in Tennessee — and the Costa Rican project eventually dissolved when it lost local political support. One early goal for the All Species Foundation is to help to complete the Hawaii ATBI within five years. It is also in discussions with the US National Park Service about helping with further inventories.

In parallel, the foundation wants to identify and promote technologies that will speed progress towards its eventual target. It is evaluating tools ranging from pattern-recognition software for quickly identifying species from digitized images, through

1.7 million organisms have been named and formally described. And that information languishes in myriad journal articles, index cards, ancient books and museum collections. Launched at a meeting at the California Academy of Sciences in September 2000, the All Species Foundation is an unusual alliance between leading taxonomists and some of the Bay Area's high-tech movers and shakers, enthused by the prospect of a web-based project with an environmental flavour. The latter include Stewart Brand, who established the online community known as the WELL, his wife Ryan Phelan, who launched the consumer health website Direct Medical Knowledge, and Kevin Kelly, a founder and now editor-at-large of *Wired* magazine. “All Species is bringing new blood and energy to the field,” observes Dan Janzen, a biodiversity researcher at the University of Pennsylvania in Philadelphia. “In all of my years, I've never met these kinds of people before,” says Terry Erwin, a taxonomist who specializes in carabid beetles at the Smithsonian Institution in Washington DC, and a member of the All Species Foundation's governing board. “It's really cool. I am usually surrounded by 12 million beetles, but I've gotten outside and talked with intellectual millionaires.”

To fulfil its mission, the foundation



Edward O. Wilson predicts that technology will rapidly accelerate the description of species.

equipment to allow rapid DNA sequencing of informative portions of a specimen's genome, to hand-held interactive versions of the 'keys' that taxonomists compile to allow naturalists to determine which species a specimen belongs to.

"Roughly 10% of species have been discovered and classified in the past 250 years," says Edward O. Wilson of Harvard University, a pioneer of biodiversity studies who favours the lower estimates for total species number, and a member of All Species' science board. "With these technological advances, we can complete the remaining 90% in one-tenth the time."

All Species also wants to encourage the creation of digital images of at least half of the type specimens — or 'holotypes' — from which each known species was originally described. The resulting 'e-types', as they are being called, would then be easily accessible on the Internet, rather than sitting in the world's leading museums and herbaria.

Index links

But the All Species Foundation isn't the only game in town. Although most taxonomists applaud the foundation's audacity, many regard its aim of documenting all living species within a generation as unrealistic. Perhaps more attainable is the goal of the Catalogue of Life, a collaboration forged in June 2001 between Species 2000 — based in Reading, UK, and Tsukuba, Japan — and the Integrated Taxonomic Information System (ITIS), based in Washington DC.



Frank Bisby sees a consortium as the best approach for taxonomy.

Within 10 years, the Catalogue of Life aims to create a federation of databases that will together describe all of the species currently known to science. Its present compilation of 18 databases includes specialist initiatives such as Antbase, FishBase, AmphibiaWeb and ILDIS, a legume-taxonomy database started by Frank Bisby of the University of Reading, who is chair of Species 2000.

The scientists behind the Catalogue of Life envision a total of up to 200 component databases. Achieving interoperability among them has the potential to be a logistical nightmare, but Species 2000 has defined a common data model for member databases to adopt. Another issue is how to deal with the fact that many species have been given more than one name. For the 260,000 species in the Catalogue of Life so far, there are 420,000 synonyms to contend with. The Species 2000 team is researching how to create a tool that allows users to be referred automatically to the correct species informa-

tion from the various alternative names and classifications.

Having joined forces to create the Catalogue of Life, the leaders of Species 2000 and ITIS are now talking with other major taxonomic players about building the project into a wider consortium. In March, representatives of Species 2000 and ITIS met in Sydney and Canberra, Australia, with organizations including the All Species Foundation and the Global Biodiversity Information Facility (GBIF), a project conceived within the Organisation for Economic Co-operation and Development's Megascience Forum.

The GBIF aims to make a range of databases on biodiversity available to researchers and policy-makers. Following the Australian meeting, its leaders are currently considering a proposal to adopt the Catalogue of Life as one of its component projects — a decision is expected in October, when the GBIF's governing board meets in San José, Costa Rica.

The All Species Foundation, meanwhile, sees its role within the emerging consortium as a driver for technology development, and in creating a web portal that will provide links to other projects' data. "It will point to all the species-level information out there," says Phelan, who is All Species' chief executive. The foundation also recently unveiled a search engine that can return taxonomic information on about 1 million species already held in web-based databases.

Bisby points out that the consortium approach worked well for another recent 'big science' biology initiative. "We are taking as our model the Human Genome Project," he says. Both the genome project and the Catalogue of Life, notes Bisby, are truly international endeavours that aim to provide a vital information resource for a diversity of researchers.

Digging deep

Working in a consortium also has the advantage of eliminating potential duplications of effort, so ensuring that each project's funds are used to the best possible effect. The GBIF currently has a total operating budget of some \$3 million per year, and the Catalogue of Life has secured a similar level of funding in grants from US, European and Japanese agencies.

The All Species Foundation, meanwhile, has high hopes of winning multimillion-dollar funding from wealthy donors. But unfortunately, the current harsh financial climate is hampering All Species' fundraising efforts.

As the various projects strive to get their funding in place, some researchers are debating whether web-based initiatives such as the



Keeping track: Dan Janzen (above) recruited parataxonomists to assess Costa Rica's biodiversity.

D. JANZEN

Catalogue of Life could become the 'official' repository of taxonomic information — currently, original species descriptions are only recognized if they appear in print (see *Nature* **417**, 17–19 & 573; 2002, and Correspondence on page 367 of this issue). Two-and-a-half centuries of tradition seem unlikely to be overturned overnight. But regardless of how this debate develops, the pioneers who are trying to reinvent taxonomy for the information age hope that, at the very least, they can help to dispel the dusty image that has done much to deter bright young biologists from entering the field.

"Old-fashioned taxonomy is less attractive to mainstream students coming into biology," says Bisby. "But we are revolutionizing the field."

Virginia Gewin recently completed an internship in Nature's Washington office.

All Species Foundation

♦ www.all-species.org

Species 2000

♦ www.sp2000.org

Integrated Taxonomic Information System

♦ www.itis.usda.gov

Global Biodiversity Information Facility

♦ www.gbif.org

The virtue of tolerance

If specific immune responses could be toned down without completely suppressing immunity, it would provide a boon for the treatment of transplant rejection, autoimmune disease and allergy. Erika Check reports.

For Betty Lewis, 1979 was a big year: she got married, was diagnosed with a progressive liver disease, and had a liver transplant. But two years later, she had a new baby, was getting divorced, and hated the way her immunosuppressive drugs made her feel. So, against her doctors' advice, she ditched the drugs. Today, she has confounded medical opinion and is still alive and well.

Immunologists are working to understand why some patients naturally achieve this state of tolerance, in which the immune system learns to accept a target that it would ordinarily attack. It's an important endeavour, because if doctors could induce tolerance at will, the prospects for transplant patients would greatly improve — immunosuppressive drugs may prevent organ rejection, but by damping down the entire immune system, they render patients susceptible to infections and cancer. Selective immune tolerance might also be used to combat debilitating allergies, or autoimmune conditions such as type 1 diabetes and multiple sclerosis.

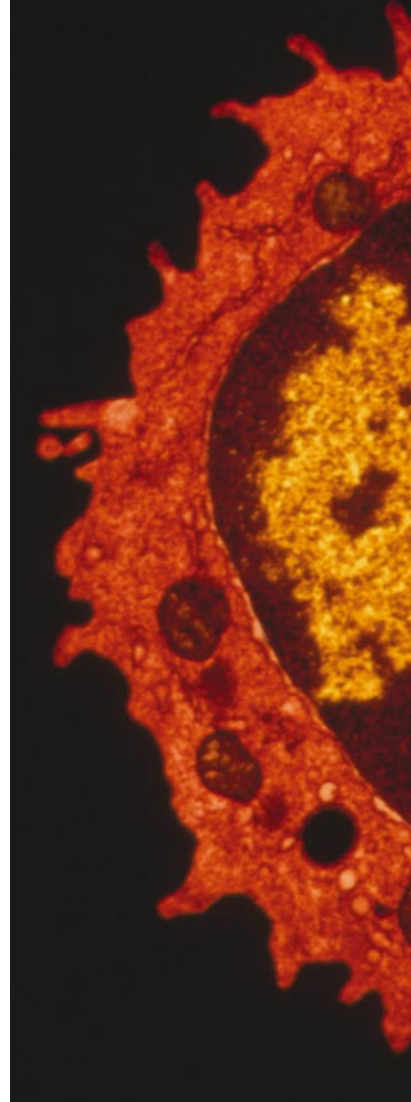
Making such treatments part of standard clinical practice is the goal of the Immune Tolerance Network (ITN), a seven-year, \$165-million project launched by the US National Institutes of Health in 1999. The network has 15 clinical trials in progress, and is gathering huge quantities of immunological data from the enrolled patients in the hope of elucidating the basic mechanisms of tolerance. But the ITN faces some tough challenges. Our understanding of the immune system is incomplete — which means that attempts to manipulate it can lead to unpredictable side-effects. And even if a tolerance therapy proves to be safe and effective, can the pharmaceutical industry be persuaded to invest in it?

"We would be incredibly excited if we could show that a very expensive drug worked in a disease that just three people in the world have, if it helped us to understand how tolerance works," observes Jeffrey Bluestone, who directs the ITN. "But a drug company would not find that a very attractive



trial to run." Nevertheless, Bluestone, an immunologist at the University of California, San Francisco, is optimistic that the ITN will identify treatments that are both clinically and commercially viable.

The key to achieving tolerance is to tame the specific cells responsible for undesired immune responses. The foot-soldiers of the



Fake ID: organ transplants would be more successful if T cells (above) in the immune system can be tricked into accepting the new tissue.

immune system are B cells, which produce antibodies that recognize a particular molecular signature, or antigen; and T cells, which likewise respond to individual antigens and attack cells bearing that signature.

Immature B and T cells are created all the time in our bone marrow, and they circulate in our blood until they encounter their specific antigen and become activated. T cells, for instance, bear receptors that allow them to lock onto 'antigen-presenting cells' (APCs) that process foreign antigens and carry them on their surface. The T cell and the APC then send each other a series of biochemical signals in a process called co-stimulation. If the T cell receives all the proper co-stimulatory signals, it becomes an activated killing machine targeted against the antigen.

The immune system needs to prevent immune cells reacting to the body's own proteins, and this is achieved through mechanisms of central and peripheral tolerance. In central tolerance, immature T cells that react with 'self' proteins are destroyed in the thymus gland in the neck. Some self-reactive T cells escape this central control, but their

activation is normally blocked by one of the peripheral tolerance mechanisms. In some cases, the T cells recognize their antigens on APCs, but do not receive the correct co-stimulatory signals. Other self-reactive T cells express receptors that cannot lock onto APCs. And some immunologists believe that a special population of regulatory T cells helps to keep self-reactive T cells in check.

Immune-tolerance therapies aim to co-opt these natural mechanisms. The most advanced work has been done in organ transplantation, where surgeons are trying to promote central tolerance by dosing transplant recipients with bone marrow from the same donors as their new organs. By establishing 'chimaerism' — a state in which the recipient's bone marrow is made up of both their own cells and those from the donor — the immune system should begin to treat the transplanted organ as self rather than foreign.

Intolerant attitudes

Autoimmune disease occurs when the mechanisms of tolerance break down. Here, researchers are trying to restore peripheral tolerance by interfering with T-cell receptors and associated molecules, or by muddling the process of co-stimulation. And to treat allergy, immunologists are trying to shift the immune response away from debilitating inflammatory reactions.

Transplant surgeons were interested in tolerance long before the ITN got under way, hoping that it might be possible for their patients to avoid a lifetime of immunosuppressive treatment. At the University of Pittsburgh's Thomas E. Starzl Transplantation Institute, surgeons have been systematically weaning liver-transplant recipients off their immunosuppressive drugs for a decade — essentially a controlled version of Betty Lewis's sudden eschewal of her drugs. Pittsburgh immunologists Angus Thomson and Adriana Zeevi are using ITN funding to run a battery of blood tests on patients who have successfully been removed from immunosuppression. They hope to find out what makes these patients different from others who have to keep taking the drugs.



Reading the signs: Angus Thomson and Adriana Zeevi have identified signals that aid tolerance.



Joined forces: bone-marrow transplants from organ donors may help to suppress rejection by the patient's immune system.

Thomson, Zeevi and transplant surgeon George Mazariegos already have some clues about how to predict which patients will become tolerant. In April, they reported a difference in the production of two cytokines — biochemical signals that help to coordinate immune responses — between 12 children given liver transplants who had been weaned off immunosuppression and 37 patients who had to stay on the drugs¹. The tolerant children were genetically predisposed to make low levels of tumour-necrosis factor- α , and high levels of interleukin-10. Such work could help doctors to define cytokine profiles to predict which patients will respond well to the withdrawal of immunosuppression.

While the Pittsburgh group examines patients who have naturally achieved tolerance, other researchers are trying to give nature a little help by using transplants of bone marrow cells from the same donor as a donated kidney. By temporarily suppressing the recipient's own bone marrow using drugs and targeted radiation, these scientists hope that the transplanted

cells will establish themselves and create a chimaeric immune system.

Samuel Strober, an immunologist who is leading an ITN-backed trial at Stanford University in California, already has promising results from a preliminary study of patients given kidney transplants by Stanford surgeon Maria Millan. His patients' blood became chimaeric, with cells from the donor accounting for up to 16% of those in their circulation. Three out of four of the patients are responding well to the phased withdrawal of immunosuppressive drugs².

Meanwhile, a group at Harvard University led by immunologist Megan Sykes and surgeon Ben Cosimi is coordinating a similar trial at multiple centres in patients with multiple myeloma, a bone-marrow cancer that leads to kidney failure in about one in five cases. This builds on a pilot study in which two such patients were completely weaned off immunosuppressive drugs³.

Surgeons are optimistic about these preliminary results — Strober's initial trial, which involved patients who were not perfectly matched to their donors, is particularly encouraging. But there is still a long way to go before bone-marrow chimaerism moves into the transplantation mainstream. First there are safety concerns — temporarily suppressing the patient's bone marrow is a harsh procedure, and there is a risk that the introduced marrow will attack the recipient's body in a potentially fatal reaction called graft-versus-host disease. Immunologists also warn that chimaerism may not prevent chronic transplant rejection, a hard-to-treat complication that can take about a decade to emerge. "The problem comes down the road," says Hugh McDevitt of Stanford University, who is not involved in Strober's trial.

Cracking chronic rejection

Immunologists are beginning to shed some light on chronic rejection, however. The T cells that attack a transplanted organ may recognize its foreign antigens directly, or require the involvement of APCs. But last year, a team led by Robert Lechler of Imperial College London reported that patients who develop chronic rejection have a stronger APC-mediated response than those who don't develop the condition⁴. Lechler now has funding from the ITN to investigate how some patients manage to keep this response in check — he suspects that regulatory T cells may be involved.

But Sykes believes the risks from chronic rejection are minimal. She points to experiments on chimaerism and kidney transplants in monkeys⁵, in which the animals have survived for seven years with healthy kidneys and no signs of chronic rejection. One of the two patients from the Harvard pilot study is also still healthy nearly five years after the transplant. "Although the caveat of chronic rejection can be raised

legitimately, I personally do not believe it is a major concern," Sykes says.

The ITN is also trying to develop new therapies for autoimmune diseases. In type 1 diabetes, for instance, T cells destroy the body's β -cells, which make insulin in the pancreas. In an attempt to halt this reaction, Bluestone and Kevan Herold of Columbia University in New York are working with an antibody to a molecule called CD3, which associates with the T-cell receptor and transmits the signal that activates the T cell.

In mice, the anti-CD3 antibody reversed symptoms of diabetes⁶, although the mechanism remains unclear. The antibody seems to reverse the activation of recently activated T cells — including those that are targeting β -cell antigens — while leaving the rest of the immune system intact.

Herold has also tested injections of the anti-CD3 antibody in a small group of newly diagnosed diabetics. First, he measured how much insulin 24 patients produced at diagnosis, then he split them into a treatment group that received the antibody, and a control group that did not. One year later, 9 of the 12 diabetics who received the antibody were still producing at least as much insulin as they did when they entered the study; but in 10 of the 12 control patients, insulin production had dropped⁷.

Fighting back

Lloyd Kasper and Randy Noelle of the Dartmouth Medical School in Lebanon, New Hampshire, hope to use a different antibody to treat multiple sclerosis — a condition in which the immune system attacks the protein myelin that coats neurons within the central nervous system. This antibody targets a molecule found on the surface of T cells called CD154, which binds to another cell-surface molecule called CD40, carried by many immune-system cells, in an interaction that is an important component of co-stimulation. In a mouse model of multiple sclerosis, the anti-CD154 antibody has already achieved promising results⁸.

But in June, Kasper and Noelle's plans hit problems when a trial they were conducting with IDEC Pharmaceuticals of San Diego was halted after concerns about one patient who seemed to develop blood clots. An earlier trial of a similar antibody to treat lupus, another autoimmune disease, was also abandoned by Biogen of Cambridge, Massachusetts, in 1999



Jeffrey Bluestone hopes to interest drug firms in immune tolerance.

these therapies are not going to rate as a high priority for pharmaceutical companies," warns Mark Feinberg, an immunologist and HIV researcher at Emory University in Atlanta, Georgia.

Bluestone admits that it is difficult to get drug companies to expand their interests beyond obvious blockbusters. For instance, the company that owns the rights to the anti-CD3 antibody — pharmaceuticals giant Johnson & Johnson — has not yet sponsored a clinical trial. So Bluestone's own lab has had to manufacture the antibody for use in the trials he has been doing. "I wish I could say on every level that people are knocking down our door, but sometimes we need to push them a bit," he says.

Firm potential

Nevertheless, Bluestone points to a recent ITN success as an example of what can be achieved if a company does climb enthusiastically on board. In March, at the annual meeting of the American Academy of Allergy, Asthma and Immunology in New York, Dynavax Technologies of Emeryville, California, reported promising results on a trial of a 'tolerizing' vaccine to treat people allergic to ragweed pollen.

Allergies are caused by inflammatory cytokines released when the immune system responds to an allergen with a 'T_H2' response, which is supposed to quell parasitic infections. Doctors have long realized that T_H2 responses can be toned down by raising an alternative T_H1 response — normally deployed against viruses and bacteria — to the same antigen. But Dynavax has managed to boost the efficiency of this approach by linking the ragweed pollen allergen to a short stretch of single-stranded DNA that helps to stimulate the desired T_H1 response. As a result, it should be possible to desensitize allergy sufferers to ragweed over six weeks, rather than six months or more.

Dynavax's clinical trials were the first to be funded by the ITN. And next year, the

after three out of 19 patients developed symptoms of clots.

Such problems serve as a stark reminder that immune-tolerance therapies remain poorly understood, and so are potentially prone to unexpected side-effects. This inherent riskiness, combined with doubts about the potential of immune tolerance to yield real money-spinners, explains the drug industry's reluctance to throw itself behind the ITN's efforts. "A lot of

company plans to begin large-scale trials aimed at gaining permission to market its vaccine. With 35 million people in the United States alone being afflicted by seasonal allergies, there is a huge potential market.

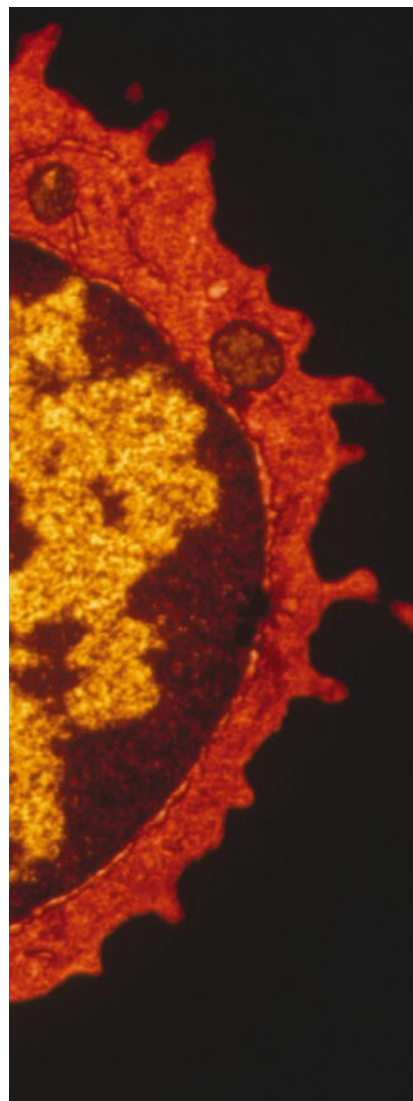
If Dynavax's experience can be repeated in other aspects of immune tolerance, Bluestone believes it will begin to draw drug companies into the field. If so, future generations of patients may be able to look forward to the robust health currently enjoyed by Betty Lewis — but by clinical design, rather than sheer good fortune.

Erika Check is Nature's Washington biomedical correspondent.

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Immune Tolerance Network

♦ <http://itn-pub.vwteam.com>



I wish I could say that people are knocking down our door, but sometimes we need to push them.

Jeffrey Bluestone

Taxonomy, at the click of a mouse

Informatics and taxonomy are working together to achieve more than either could alone.

Sir — We welcome your Opinion article “Genomics and taxonomy for all” (*Nature* **417**, 573; 2002) and H. C. J. Godfray’s creative ideas for modernizing taxonomic practices in his Commentary “Challenges for taxonomy” (*Nature* **417**, 17–19; 2002; see also News Feature pages 362–363). Taxonomy provides the reference system for all biology, so organizing, updating and streamlining the process of bringing taxonomic information onto every desktop at the click of a mouse are urgent priorities. The first substantial steps in this direction are already being taken, as mentioned by Godfray, and there is more to follow.

First, Species 2000 (www.sp2000.org) and the Integrated Taxonomic Information System (www.itis.usda.gov), two major players in creating an electronic global framework for taxonomy, joined forces last year in the Catalogue of Life consortium and are now making rapid progress with a catalogue of all known organisms. They have invited other organizations to join them in constructing a complete and freely available web-based synonymic index of species and associated data. The 2002 Catalogue of Life now lists 260,000 species (860,000 names, including synonyms and common names) on CD-ROM and on the Web. This programme is community-wide — the sources of this knowledge are the participating taxonomic database programmes embedded in the taxonomic profession and supported by systematics institutions, projects and individuals around the world.

Second, the Global Taxonomy Initiative (GTI) of the Convention on Biological Diversity (see decision VI/8 of COP 6 at www.biodiv.org/decisions) is building

taxonomic capacity and making taxonomic information available to help implement the convention. A programme of work for the GTI was formally adopted at the Hague in April, providing political endorsement and a framework for national, regional and global projects to develop the knowledge base. Funding comes from various sources, including the Global Environment Facility.

Third, the Global Biodiversity Information Facility (GBIF; www.gbif.org) aims to develop an interoperable network of biodiversity databases and information technology tools for users to explore the world’s vast quantities of biodiversity information for economic, environmental and social uses. GBIF is developing standards for interoperability, digitizing biodiversity data, helping to complete the Catalogue of Life, and improving global taxonomic and biodiversity informatics capacity. GBIF is also developing a portal to provide specialized search engines for accessing digitized, georeferenced specimen data from the world’s herbaria, museums and other natural history collections. In this it is working closely with the Catalogue of Life consortium, GTI and other taxonomic organizations.

Fourth, we endorse Godfray’s suggestion that species descriptions, images and a platform for publication and debate should be provided on the web. These online community-building features are not yet generally available for the Catalogue of Life, but individual contributing systems within Species 2000 are already experimenting in this area. ILDIS LegumeWeb (www.ildis.org) has the first ‘community taxonomy’ created and maintained by worldwide contributors to a

global database. FishBase (www.fishbase.org) and AlgaeBase (www.algaebase.org) are experimenting with web-based facilities for users to offer images, data on new species and so on. At present none of these publishes the original diagnoses of new species, but the infrastructure that could provide this facility is already in place. GBIF is similarly planning a digital library of biodiversity literature and a ‘Species Bank’, to include the collaborative, online community-building features proposed by Godfray.

All these activities are in tune with Godfray’s proposals and your Opinion article. The Catalogue of Life and the GBIF are each funded at about US\$3 million a year and we should see real progress over the next three years. Several projects under the aegis of the GTI have been funded, although substantial further resources will be needed for all three initiatives. But the excellent news is that interest in taxonomy is reawakening, and the taxonomic and informatics communities are working together to accomplish much more than either could achieve separately.

Frank A. Bisby*, Junko Shimura†, Michael Ruggiero‡, James Edwards§, Christoph Haeuser||

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Strong case for neutrons

Sir — On 18 July you reported (*Nature* **418**, 262; 2002) a review of major facilities and an assessment of the European Spallation Source (ESS) by Germany’s science council, the Wissenschaftsrat. As your report says: “The council endorsed an assessment of the ESS by a subcommittee ... that the demand for neutrons does not justify the estimated 1.4-billion-euro (US\$1.4-billion) total investment in an advanced neutron source.” This assessment reflected neither the consensus view of the subcommittee nor the opinion of most members. As members of this subcommittee, we have written to the chair of the Wissenschaftsrat with our concerns, paraphrased below.

“Contrary to the impression given by

the draft subcommittee report presented to the Wissenschaftsrat, most of the committee judged the scientific case for the ESS to be very strong. The research opportunities for many fields of applied and fundamental science, such as solid state physics/chemistry, particle physics, biology and engineering, were seen as novel and outstanding. Critical remarks by some members were confined to the area of polymer physics/chemistry.

“Further, contrary to the summary of our deliberations in the Wissenschaftsrat’s report, we do not see reason for concern about the timeliness of the scientific programme. We agree with the Japanese and US research communities, who have successfully sought long-term funding for high-intensity spallation sources, that

experiments with neutrons will play a leading role across the sciences for the foreseeable future.

“The Wissenschaftsrat’s statement on the cost of neutrons relative to other techniques is not based on input from us and we do not believe it would be supported by a comprehensive analysis.”

B. Keimer

Max Planck Institute for Solid State Research, 70569 Stuttgart, Germany

Other signatories to this letter:

H. Dosch Max Planck Institute for Metals Research, Stuttgart, Germany

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H. Fuess Technical University of Darmstadt, Germany

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E. Sackmann Technical University of Munich, Germany

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The question of animal rights

Should cognitive ability determine which animals deserve legal rights?

Drawing the Line: Science and the Case for Animal Rights/Unlocking the Cage: Science and the Case for Animal Rights

by Steven M. Wise

Perseus Press: 2002. 336 pp/320 pp.

\$26 (hbk)/£10.99 (pbk)

Diana Reiss

Drawing the Line is a provocative and disturbing book. Steven Wise, a lawyer who used to teach animal-rights law at Harvard University, is a former president of the Animal Legal Defense Fund and a leading advocate for non-human animal rights in the United States. In his previous book, *Rattling the Cage*, he argued that apes, specifically chimpanzees and bonobos, are entitled to basic legal rights because of their social complexity and advanced mental abilities.

In his new book, *Drawing the Line/Unlocking the Cage*, Wise explores the cognitive abilities of several species including apes, bottlenose dolphins, elephants, dogs, parrots and honeybees and discusses their eligibility for legal rights based on their higher cognitive abilities. He argues that a minimum level of autonomy — the ability to desire, to act intentionally and to have some sense of self — is sufficient, whatever the species, for the right to dignity and to justify the basic legal right to bodily integrity.

Wise considers whether there are species that demonstrate 'practical autonomy' and deserve the same rights as humans. He views practical autonomy as the crucial factor in deciding where to draw the line, and questions where, and more importantly why, we draw the line between human and non-human animal rights. The book presents a compelling and cogent review of what Wise sees as the historical, legal, economic, political, religious, physical and psychological issues and obstacles to establishing the rights of animals. Wise explains that he has taken this approach because other arguments for the protection of non-human animals in courts of law have failed.

Primarily concerned with how the scientific study of animal cognition can inform animal-rights law, Wise reviews many of the leading studies on animal cognition and social behaviour. He has interviewed many prominent scientists in the fields of cognitive psychology, cognitive ethology and animal behaviour, and reviews several field and laboratory studies of social behaviour and cognitive abilities in non-human animals. From his assessment of this research, he assigns a numerical score, or "autonomy value", to each species and places them in one of four



Rights issue: Steven Wise argues that bonobos, like humans, should have a legal right to dignity.

categories. Only species achieving the rank of "category one" are candidates for rights. He concludes that, according to his rating scale and our present state of knowledge, apes and dolphins attain category one and deserve rights. Species such as dogs and African grey parrots are designated to category two, as their level of practical autonomy is less certain, and should be considered for some rights on the basis of the "precautionary principle". He urges us to err on the cautious side and give these animals the benefit of the doubt regarding their cognitive abilities.

Although Wise presents a mostly reasonable and readable review of cognitive studies on non-human animals, reader beware, as some of the scientists and methodologies described in the book are incorrectly represented or misquoted. Ironically, he relies on the ground-breaking research of cognitive scientists and then, on occasion, turns on the same scientists for studying captive animals. It seems obvious, but important to note here, that without such careful and humane studies of animals we would understand very little of their cognitive abilities.

Despite numerous field and laboratory studies, cognitive research in non-human animals is still in its infancy, and it seems premature to draw firm conclusions regarding absolute cognitive indices upon which the categories are based. In addition, should specific levels of cognitive ability really determine which organisms deserve rights and humane treatment and which do not?

If Wise's ultimate goal is to rally public

support for legal rights for non-human animals, his extreme argument may in fact undermine a much stronger argument that he could be making: that humane scientific research on animal intelligence and behaviour should inform and exert a greater influence on legal and scientific policies regarding animal protection, welfare and conservation. Historically, practices of animal husbandry have depended on and have tracked advances in physiological research; the result has been improved animal care. So advances in animal cognitive research should similarly lead to revisions in animal-care policies, and guide and influence policy decisions that affect the protection of individual animals and conservation efforts on the species level.

An enormous problem that the author repeatedly acknowledges throughout the book is that the use of animals is inextricably interwoven into human activities. Yet he devotes little time to a more global perspective focusing on issues that face animals worldwide, such as habitat destruction, poaching, whaling, the bushmeat trade, the condition of animals within the food industry, and even the abuse and neglect of domestic pets.

In the end, those of us who work for the humane treatment of individual animals or the protection of species know that there are always competing interests. When faced with issues of animal rights and wrongs, we are the ones in the position of power and decision-making. Ultimately, where and when we choose to draw the line on how we treat other

animals will be decided either in courts of law, culturally or by each of us personally, depending on the circumstances. This is an important book to read because it challenges all of us to rethink where and why we draw the line. ■

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More on animal cognition

The Alex Studies: Cognitive and Communicative Abilities of Grey Parrots

by Irene Maxine Pepperberg
Harvard University Press, \$18.95, £12.95 (pbk)

Minding Animals: Awareness, Emotions, and Heart

by Marc Bekoff
Oxford University Press, \$27.50, £18.99

Replication at the speed of thought

The Electric Meme: A New Theory of How We Think

by Robert Aunger
Free Press: 2002. 400 pp. \$27

Eörs Szathmáry

The 'm-word' is regarded by many in polite intellectual society as belonging to that other set of four-letter words. Some think that memes are at best cultural replicators with a loose analogy to genes; to others they are just a fashion that will fade away; yet others think they are a prime example of hubris, with biologists trespassing into the social sciences as if responding to attacks by deconstructionists. It isn't clear whether there is a single fact of life that memes, rather than other mental constructs, have successfully explained so far. The spread of the meme concept has infuriated many; some might say that the only good example of a meme is the meme concept itself. But the latter cannot be true: there are either zillions of memes, or none at all.

Many memeticists — scholars from different disciplines dealing with memes — tend to think that memes are best identified at the level of directly observable items, such as behavioural patterns, utterances, texts or artefacts. This idea has a certain operational appeal, and lends itself to describing meme replication in terms of social learning, such as imitation. The informational meme corresponds to the informational gene, for which it is the genetic information that matters, rather than the DNA molecule itself.

Yet problems abound. Where is memetic information primarily stored? In the brain, surely. But how are memes stored there? Hand-waving may be grossly insufficient. Aunger begins his fascinating book by pointing out that clarifying the material basis of inheritance has profoundly deepened our knowledge about genetic phenomena. Indeed, some phenomena are so puzzling at the phenomenological level that trying to understand them without the underlying replication mechanism is hopeless — think of jumping genes, for example.

Aunger considers neural memes rather than abstract memes. He identifies memes as dynamic neural activity patterns (states) that can be replicated, primarily within the brain. In so doing he makes a connection between ideas of intracortical replication — such as Calvin's Darwin machine, in which a kind of fax-like copying of neural states is postulated to occur in hexagonally arranged columns — and memes as replicatable items. Such replication in the brain could have been selectively advantageous for reasons of redundancy and problem solving. Then, in some animals including dolphins and primates, memes developed the ability to hop from brain to brain — the 'classical' case for abstract memetics. Later, in human populations, artefacts appeared as extended phenotypes of neural ('electric') memes, which then co-evolved with memes. With this we are in the twenty-first century, with advanced information technology all around us. But are we?

With regard to Aunger's broad picture, I think that we are. I agree with many of his ideas, and consider some of them to be truly novel and fascinating. For example, the idea that memes are primarily neural phenomena at the millisecond scale should open up a field of relevant neurobiological investigation. Hopefully this will enable the fleshing out of Aunger's rather telegraphic account of how millisecond memes are maintained and replicated. I also agree with Aunger that DNA replication and meme replication are fundamentally different processes, which isn't clear from the phenomenology. We

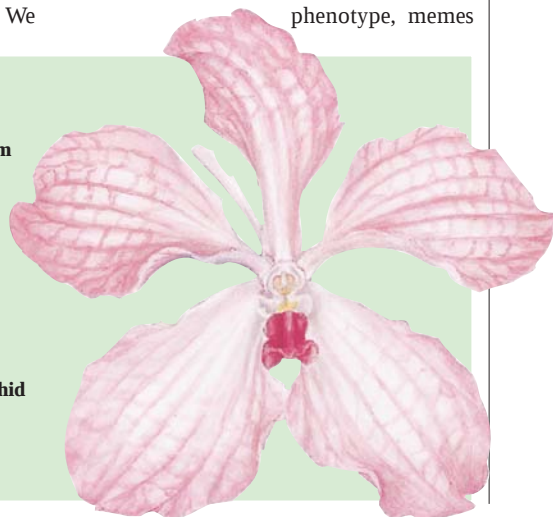
also share the view that replications should always be analysed in terms of a formal chemistry. From this it is clear that the replication of memes is more similar to the replication of prions, which Aunger aptly calls conversion, than of genes, and that more than one code is involved in memes hopping between individuals.

Where are the disagreements, then? Aunger identifies the basic difference between animal and human cultures in the artefacts of the latter. I agree with the importance of meme-artefact coevolution, but it strikes me that Aunger almost misses out language. This is a deficiency, because it has been argued at length elsewhere: human language is a hereditary system allowing for the spread of replicators with practically unlimited hereditary potential. It is an excellent subject for memetics, partly because the memes that underlie language are a prime example of memes affecting other memes. For humans, language memes are like genes coding for elements determining the replication and expression of genes. And if the neural foundations are important, language could be the case where millisecond memes will be nailed down, perhaps by advanced (not yet available) brain imaging.

I think that Aunger's treatment of replication between individuals is rather misleading, or perhaps not radical enough. To my mind, memes are phenotypic replicators, reconstructed in other minds by conversion from pre-existing memes, the criterion being sufficient phenotypic convergence. Aunger is right to say that signals (such as words) by themselves are neither memes nor memetic phenotypes, but he seems to miss the point that a set of signals associated with a meme is in fact its phenotype. It is this set, rather than any particular signal, on which the transmission of a meme to one brain to another is generally based. The radical conclusion that I hold (in agreement with John Maynard Smith) is that, whereas genes are weismannian replicators, with no flow of information back from the phenotype, memes

Floral print

The flowers of the celebrated 'blue' orchid, *Vanda coerulea* 'The Node' (right), vary from mauve-white to lavender blue. The orchid was heavily collected from the wild before the development of equally popular hybrids. The Royal Horticultural Society started its own collection of orchids in the 1830s and has commissioned watercolour paintings of many of the prize-winning blooms as judged by its Orchid Committee. A selection of these, together with other orchid illustrations in its possession, has now been published in *Orchids* by Mark Griffiths (Scriptum Editions, £45).



are lamarckian ones, relying on reverse encoding from the phenotype, as if genetics canonically included something akin to reverse translation, or at least some other means of inheriting acquired traits. The immediate consequence is higher variability and the potential for cultural evolution to be much faster than genetic evolution. ■

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Running through the mind

Lore of Running, 4th edition
by Tim Noakes
Oxford University Press: 2002. 1,277 pp.
£27.99

Norman Myers

Following Khalid Khannouchi's victory in April's London Marathon, in which he broke the men's world record, there was renewed speculation that male athletes would eventually trim five minutes off the record and complete the course of 26.2 miles inside two hours. The experts promptly declared it exceedingly unlikely, if only because it would mean running four consecutive sets of 10 kilometres in times that individually would be beyond all but the best of today's athletes. So it's a tall order — but impossible?

For decades it was considered beyond human capacity, virtually in physiological principle, to run a mile inside four minutes. Since Roger Bannister achieved it in 1954, hundreds of others have breached the barrier, which was thus shown to be psychological rather than physiological. A few athletes can now run two miles at almost Bannister's speed. And some African athletes run several miles in training at almost this pace.

Shortly after his legendary mile, Bannister asserted that: "Though physiology may indicate respiratory and circulatory limits to muscular effort, psychological and other factors beyond the ken of physiology set the razor's edge of defeat or victory and determine how close an athlete approaches the absolute limits of performance." This statement is almost fifty years old, yet most research is still directed at the physical side of running.

It is the psychological aspect — the complex chemistry of ambition and competitiveness — that absorbs Tim Noakes, director of the Department of Sports Medicine at the University of Cape Town, South Africa, and one of the world's leading scientists in endurance sports. This book is a thoroughly revised and expanded version of his 1985 original. A large portion of this fourth edition presents new findings and



Beat the clock: Roger Bannister and Khalid Khannouchi (inset) both broke world records.

analyses on the "psychic" side of endurance events — not just running, but swimming, rowing and cycling.

He provides a detailed evaluation of the thesis that the brain is in ultimate control of the body, and that fatigue is part of a regulatory process to prevent excessive physiological effort. In other words, the body has a safety-first mechanism that rings warning bells well before the onset of actual danger. However exhausted a runner may feel, he is likely to be a fair way from exhausting all his physical potential. The trick is to close the gap through targeted training, reflecting the best sports science available.

Not surprisingly, Noakes is particularly illuminating in his sections on "What makes a winner?" World-class runners are all at virtually the same peak of physical fitness, so the medal goes to whoever most wants to win and best deploys the neurological commitment that reflects the wanting. Among sports-science books, this one does more than most to further our understanding of what makes up the endurance athlete who is also a superb competitor. This applies not only to the attributes of Olympic champions, but also to those of talented amateurs.

The book is illuminating on more conventional aspects of running, notably the physiology and biochemistry of energy systems, the cardiovascular and anaerobic models of athletic performance, and the principles of training, especially for elite athletes. Interspersed throughout the book are profiles of leading runners such as Roger Bannister, Josiah Thugwane, Elana Meyer, Bruce Fordyce and other world-class performers, plus a wealth of insights into the training regimes of athletes from northern, eastern and southern Africa, North America, Western and Eastern Europe, Japan, Australia and New Zealand (although there is

little on Nepal, Bolivia and other high-altitude countries that theoretically should be producing winners). The presentation of so much detail on the brain's role in determining physical performance, juxtaposed with Noakes' expertise as an exercise physiologist, makes this a unique compendium of the latest scientific thinking.

All this is presented in a genial style that makes this long book readable from start to finish. Noakes views running as much more than putting one fleet foot in front of the other. He sees it as a sport in which it is possible to be completely alone, and where overcoming physical discomfort provides the means for personal growth: "Running has influenced my life by teaching me who I am, and, equally importantly, who I am not. Even in the most crowded races, the point is reached when fatigue drives each of us back into ourselves, into those secluded parts that we discover only under times of such duress and from which we emerge with a clearer perspective of the people we truly are." ■

Norman Myers is Honorary Visiting Fellow, Green College, Oxford University, Upper Meadow, Old Road, Oxford OX3 8SZ, UK. He advises athletes in Kenya and California, and is a former holder of the Kilimanjaro record (ascent and descent from 5,500 feet to 19,340 feet during daylight hours).

correction In the review of Benjamin Yandell's *The Honors Class: Hilbert's Problems and their Solvers* (*Nature* **417**, 693; 2002), the picture caption should have identified David Hilbert as being "bottom row, right".

Soft water paths

Peter H. Gleick

Not a moment too soon, the world is awakening to the need to rethink fundamentally the way freshwater resources are distributed, managed and used. In an era of technological breakthroughs and the wonders of the information revolution, millions die each year from preventable water-related diseases, and hundreds of millions more suffer from debilitating illnesses. Despite massive investment and effort, by the end of the twentieth century 2.4 billion people (more than lived on the entire planet in 1940) lacked sanitation services of the standard available to most citizens of ancient Rome. More than a billion people still lack adequate, clean drinking water.

No single factor is responsible for this deplorable state of affairs. Weak governments, limited capital, misdirected development efforts and rapid population growth are just some of the many contributing problems. Two paths lie before us: a 'hard path' that will rely almost exclusively on centralized infrastructure to capture, treat and deliver water supplies; and a 'soft path' that will complement the former by investing in decentralized facilities, efficient technologies and policies, and human capital. This soft path will seek to improve overall productivity rather than to find new sources of supply. It will deliver water services that are matched to the needs of end users, on both local and community scales.

The traditional hard-path approach has produced, and will continue to produce, enormous benefits, such as clean water supplies, irrigation and improved human health. But increasingly it is also spawning ecologically damaging, socially intrusive and capital-intensive projects that fail to deliver their promised benefits.

The soft path requires governments,

communities and private companies to collaborate to meet water-related needs, rather than merely to supply water. The productive use of water can be improved by rational application of technology and economics, and by decision-making at the right scale. Ecological health must be considered a fundamental component of water policy. This contrasts with the unshakeable belief of most policy-makers that large, centralized water systems are the only way to meet unrelenting growth in demand, and that such demand is an inevitable outcome of growth in population and gross domestic product (GDP).

Yet the link between economic growth and water demand can be broken. From 1900 to the mid-1990s, the GDP of the United States rose by a factor of 20. Total water withdrawals increased more than tenfold between 1900 and 1980, but then began to decline. By 1995, per-capita withdrawals had dropped by 20% relative to the 1975 figure. A similar pattern can be seen in other industrialized countries, and even some developing nations are decoupling water use from economic and population growth.

Two factors are driving this decoupling. The first is a broad improvement in productive use of water — we are improving our ability to provide the goods and services we want with less water. The second is a shift away from water-intensive industries.

The efficiency of water use can be improved further through technological innovations, new policies and simple improvements in operations. In the 1930s and 1940s, steel production for example typically consumed up to 200–300 tonnes of water per tonne of steel. Today just 3–4 tonnes of water are required to do the same job in the most efficient plants.

Many economies have shifted production away from water-intensive industries

Water management

The soft path seeks to improve the overall productivity of water use and deliver water services matched to the needs of end users, rather than seeking sources of new supply.

such as steel production or chemical manufacturing, to industries such as service provision, telecommunications and computing. This has fuelled a further divergence between economic production and water use. Hong Kong and California, for example, have doubled their economic productivities per unit of water use over the past 30 years.

Improving agricultural productivity is critical because this sector accounts for more than two-thirds of human water withdrawals. Efficient irrigation technologies can help to boost food production with a limited water supply. Precision irrigation, soil-moisture monitoring and laser-levelling of fields, for example, have the potential to double productivity per unit of water for many crops. Shifting from conventional surface irrigation to drip irrigation in India has increased overall water productivity by 45–255% for crops as diverse as banana, cotton, sugar cane and sweet potato. Yet worldwide, only about 1% of irrigated land is irrigated by precision systems.

Many uncertainties remain about the physical, practical and economic potential of soft-path alternatives. But these uncertainties can be reduced through modest investment in data collection and analysis, consistent regulations and standards, and appropriate application of economics. The soft path will not be easy to follow. It will require institutional changes, new management tools and skills, and a greater reliance on actions by many individual water users rather than a few engineers. Yet when compared with the growing cost to society of continuing down the hard path, it is evident that a new way of thinking about our scarce water resources is long overdue. ■

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FURTHER READING

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Lessons from the past: over 2 billion people now lack the sanitation services enjoyed in ancient Rome.

BETTMANN/CORBIS

Much binding in the lab

Andrew D. Hamilton

Application of a concept drawn from two areas of macromolecular chemistry shows how artificial binding sites that resemble those found in globular proteins can be made.

Chemists have often looked with awe at the dazzling array of biochemical functions and chemical transformations in nature. So, if nature can do it, why can't we? Part of the answer is, of course, the many millions of years over which natural selection has had time to optimize an enzyme active site or a protein–DNA interface, compared to the usual term of a research grant or the four or five years available to PhD students. Synthetic chemists are, nonetheless, on the case, and a clever way of creating molecular-recognition systems is described by Zimmerman *et al.*¹ on page 399 of this issue. They have taken an important step in establishing a family of synthetic compounds that can be easily prepared and show recognition properties that bear comparison to those of antibodies.

The exquisite recognition of an antigen by its antibody is just one instance of the remarkable chemical control achieved in biology. Regulation of gene expression by transcription factors and the seemingly effortless execution of difficult reactions by enzymes are other examples. Yet these transformations are carried out with a very limited set of building-blocks — just four nucleotide bases and 20 amino acids. Nature can fashion the correct chemical microenvironments for binding a substrate or cleaving a bond by simply folding a string of amino acids, but synthetic chemists have yet to design and create an equivalent receptor or catalyst. There are essentially no examples of synthetic molecules that bind to an antigen with the strength or selectivity of an antibody, or that split the bonds between amino acids at physiological pH and temperature like the digestive enzyme trypsin. But although chemists don't have the time that nature has had, they do have a large array of synthetic reagents and potential building-block designs for constructing functional mimics of proteins or nucleotide chains.

Using entirely synthetic components, Zimmerman *et al.*¹ have created a globular molecule containing an interior recognition site that can bind to certain modified porphyrins and differentiate between closely related analogues. Porphyrins are a family of large organic molecules that occur naturally (for instance, as a component of haemoglobin) but are commonly used in synthetic chemistry. The key element in Zimmerman and colleagues' approach is

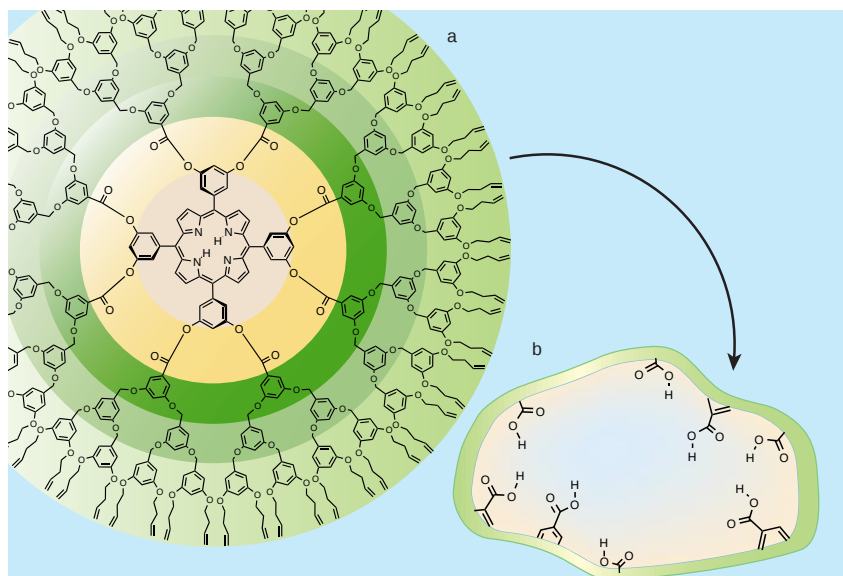


Figure 1 The approach used by Zimmerman *et al.*¹ to create a synthetic globular structure enclosing a molecular recognition site. **a**, Dendrimer construction. The template porphyrin with its four phenyl groups (beige) is linked by ester bonds (yellow) to three generations of benzene derivatives (green). This is a two-dimensional depiction of a three-dimensional process, and with only partial representation of the benzene groups. The particular innovation described by Zimmerman *et al.* is crosslinking of terminal alkenes on the third generation, which stabilizes the structure and allows the central porphyrin to be removed by cleaving the ester bonds. **b**, The result is a globular molecule, containing a cavity of a specific size and shape, lined by eight carboxylic-acid groups, that serves as a well-defined binding pocket for other modified porphyrins. (Figure based on Fig. 1 of ref. 1.)

the merging of two areas of macromolecular chemistry, dendrimers and molecularly imprinted polymers.

Dendrimers have long been seen as a discrete branch of polymer chemistry, where the macromolecule is grown out from a central core to create a roughly spherical compound with a single molecular weight². But dendrimers suffer from their design. They tend to be unduly flexible and have little interior space in which to form an active site with well-positioned binding groups. On the other hand, molecularly imprinted polymers often contain effective binding pockets within the confines of a stable polymer network. The pockets are formed by carrying out the polymerization around a molecular template which is then removed from the matrix³. The resulting cavity possesses the shape (and complementary chemical characteristics) of the template and can be used to bind related chemical structures. But molecularly imprinted polymers can be limited by the chemical heterogeneity of the polymer in terms of size and binding-

site structure, as well as restricted solubility.

Zimmerman *et al.*¹ have designed a single molecular template from which they construct a dendrimer by attaching functional groups to create a divergent framework. The template is a porphyrin with four phenyl groups, each of which bears two hydroxyl groups (Fig. 1a). All eight hydroxyls are linked through easily cleaved ester bonds to a wedge-shaped dendrimer segment composed of three generations of benzene derivatives⁴. The third-generation component contains terminal alkenes. The result is a classical, almost spherical dendrimer with 64 alkene groups on its surface.

The significance of the alkenes is that they contain carbon–carbon double bonds, which can be broken and re-formed as new double bonds between neighbouring alkene groups. It is here that Zimmerman and colleagues' approach comes into its own. Using a catalyst — Grubbs' olefin metathesis catalyst⁵ — the peripheral alkenes are crosslinked to stabilize the shape of the dendrimer that

has been formed around the porphyrin template. Analysis with nuclear magnetic resonance, mass spectrometry and chromatography confirmed that virtually every alkene had reacted with a neighbour. The whole superstructure is then sufficiently stable to allow the porphyrin template to be removed by cleaving the ester bonds. This leaves behind a cavity, lined with eight carboxylic-acid groups, within the interior of the globular molecule (Fig. 1b). The size and shape of the cavity betray its origins from the porphyrin template.

The authors show that the dendrimer host binds strongly to a test substrate — a porphyrin with four pyrimidine groups, with a total of eight basic nitrogen atoms in appropriate positions to form hydrogen bonds to the carboxylic-acid groups in the cavity. There is subtlety in the recognition, suggesting a well-defined binding pocket. The original porphyrin template with its eight hydroxyls does not itself bind because the combined sizes of a carboxylic acid and a hydroxyl group make too tight a fit.

However, the design is not perfect. There is flexibility in the dendrimer host structure that leads to intramolecular hydrogen bonds between carboxylic acids in the binding pocket. This results in an affinity that is simi-

lar for substrates irrespective of whether four or eight hydrogen bonds are formed. Also, the lack of discrimination among different isomers of a tetrapyrrolyl porphyrin suggests that the carboxylic-acid groups can move within the binding cavity and contact the nitrogen atoms in different positions.

Nonetheless, the overall concept outlined by Zimmerman *et al.* is a promising one. The idea of using a substrate (or at least a close analogue) as the template for the synthesis of its own host will find many future applications, for example in drug delivery and catalyst design, and in devising novel separation strategies. Nature still has an edge in terms of affinity and selectivity, but Zimmerman and colleagues' approach will now permit the construction of artificial binding sites that resemble those in globular proteins. ■

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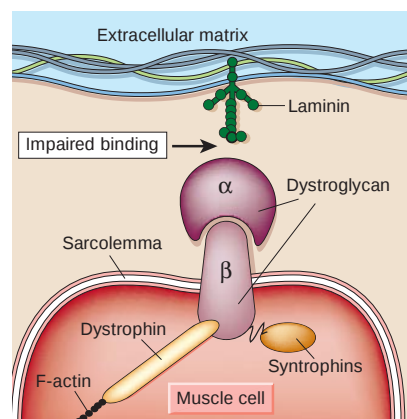


Figure 1 Congenital muscular dystrophy and α -dystroglycan³. In muscle, dystrophin binds both F-actin in the cytoskeleton and the 'dystrophin glycoprotein complex', which includes dystroglycan. α -dystroglycan is a secreted component that lies outside the muscle cell. To function properly, it must be glycosylated — have sugar groups attached — and bind both β -dystroglycan in the cell's membrane, the sarcolemma, and proteins in the extracellular matrix such as laminin. Failure of glycosylation impairs binding to the extracellular matrix, destroying the muscle fibre over time.

stage. So there have been hints that other proteins cannot substitute for dystroglycan, and that even reduced dystroglycan activity might underlie the human disorders.

Michele *et al.*³ now provide evidence that reduced (hypo) glycosylation of α -dystroglycan is involved in the two diseases, as well as in a naturally occurring mouse mutant, the myodystrophy (*myd*) mouse. Muscle biopsies from patients with MEB and FCMD revealed normal patterns of β -dystroglycan but no glycosylated α -dystroglycan. In electrophoresis, the core α -dystroglycan protein showed a shift in mobility, interpreted as a change in apparent molecular weight due to loss of sugar groups. Furthermore, the change in mobility of the α -dystroglycan component was identical for the MEB and FCMD samples, implying that the different glycosyltransferases mutated in these diseases affect the same sugar residues on α -dystroglycan.

The authors further show that the hypo-glycosylated α -dystroglycan from patients was impaired in binding proteins such as laminin, agrin and neuroligin — all of which are components of basement membrane, the specialized sheet of extracellular matrix that surrounds muscle and other cells. Similar biochemical abnormalities were evident in both the muscle and brain of a *myd* mouse with a mutation in a gene — the *LARGE* gene — which again is thought to encode a glycosyltransferase⁵. Finally, Michele *et al.* find that defects in neuronal migration in the *myd* mouse brain are like those seen in MEB and FCMD patients.

In sum, Michele *et al.*³ show that muta-

Neurobiology

Full circle to cobbled brain

M. Elizabeth Ross

A biochemical link between certain congenital muscular dystrophies and the associated brain malformation known as cobblestone lissencephaly has been elusive. But it looks as if that link has been found.

Muscular dystrophies are genetic diseases that cause progressive muscle weakness. The best known is that described by Duchenne, which affects boys and is evident from about five years of age, and which results from mutations in the gene encoding a protein called dystrophin. Another subclass is the congenital muscular dystrophies, where muscle weakness is apparent at birth or shortly afterwards. Two of these for which gene mutations have been found are muscle-eye-brain disease (MEB) and Fukuyama congenital muscular dystrophy (FCMD). Children carrying the faulty MEB or FCMD genes^{1,2} suffer from both muscle weakness and 'cobblestone lissencephaly', in which a flaw in neuronal migration results in a brain with a bumpy, cobblestone appearance and loss of the normal folding pattern.

How the two very different muscle and brain defects arise in the same patient has not been known. Now, however, companion papers by Michele *et al.*³ and Moore *et al.*⁴ (pages 417 and 422 of this issue) describe an impressive array of data that points to a

common mechanism. To function properly in muscle, dystrophin has to form complexes that include two components, α and β , of another protein, dystroglycan. Each of these has to be appropriately modified by glycosylation — the addition of sugar molecules by glycotransferase enzymes. The β -dystroglycan in the membranous sheath of a muscle cell, the sarcolemma, binds α -dystroglycan; in turn, α -dystroglycan binds to proteins such as laminin in the extracellular matrix. The two papers provide evidence that the defect underlying muscle weakness and brain abnormalities in both MEB and FCMD is disrupted glycosylation of α -dystroglycan (Figs 1 and 2).

The MEB and FCMD genes both have similarity to genes known to encode glycosyltransferases, although it was unclear which substrates of these enzymes are relevant to the congenital dystrophies. Likely candidates, however, lie in the dystrophin-dystroglycan complex. Mutations in dystroglycan have not hitherto been associated with human disease. But a 'knockout' of dystroglycan in mice proves lethal at the embryo

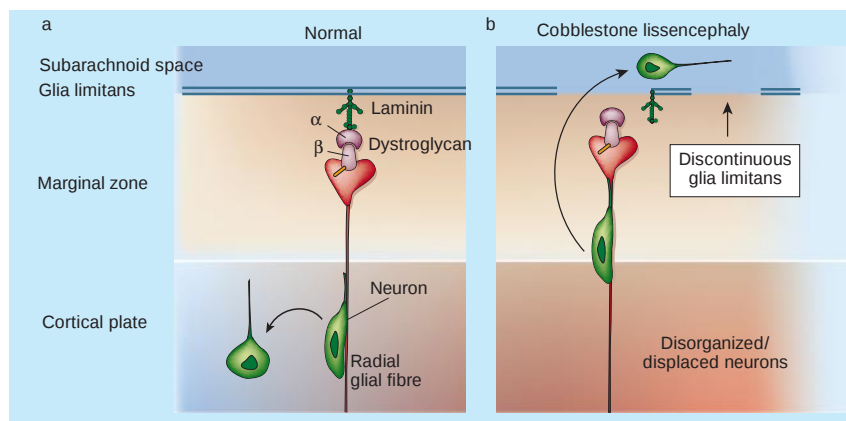


Figure 2 Cobblestone lissencephaly and α -dystroglycan⁴. In brain development, the dystrophin–dystroglycan complex is present in both of the two principal types of brain cells, glia and neurons. **a**, During normal development, the top of the cerebral cortex is defined by a basement membrane — the glia limitans — at the end of the radial glial fibres that guide neuron growth. This prevents neurons that form the marginal zone and cortical plate, two layers of the cortex, from migrating out of the brain proper and into the subarachnoid space. **b**, In cobblestone lissencephaly, failure to glycosylate α -dystroglycan is associated with gaps in the glia limitans, and failure of neurons to organize themselves within the cortical plate and their migration into the subarachnoid space. It remains to be seen whether neuronal motility is also directly affected.

tions in three different glycosyltransferases result in similar biochemical abnormalities in α -dystroglycan, with associated changes in muscle tissue. Moreover, the glycosyltransferase mutation in *myd* mice also results in a neuronal-migration defect that would be expected of a congenital muscular dystrophy in which the brain is affected. One consequence in the mice is a disrupted basement membrane — the glia limitans — of the glial cells that provide essential physical support for neurons in the brain.

Moore *et al.*⁴ make the story even more convincing. They took the next logical step: seeing whether knocking out dystroglycan, the proposed target of the glycosylation defect, has the expected effects in the brain. Mice with complete loss of the dystroglycan gene die as embryos, so Moore *et al.* made a 'conditional' knockout that allowed them to inactivate the gene's expression in glia and brain neurons only.

The result is a viable mutant with normal muscle but brain malformations similar to those seen in MEB and FCMD, and also in Walker–Warburg syndrome, another congenital muscular dystrophy. Most notably, the glia limitans is affected, presumably permitting the overextended migration of neurons in the developing brain (Fig. 2). This is the most important diagnostic feature of cobblestone lissencephaly. The mice also lack the usual fissure between the brain's hemispheres, a characteristic of Walker–Warburg syndrome, and suffer from an overabundance of glia. This last effect could arise in reaction to the loss of basement-membrane integrity, and the consequent inflammatory response, or could be due to the altered development of neurons or glia, or both.

Dystroglycan may also work in the neu-

romuscular junction⁶, the structure at which neurons and muscle cells connect and which is similar to the synapses that connect neurons in the brain. So Moore *et al.*⁴ went on to look at synaptic function in their knockout mice. Their results from electrophysiological recordings on brain preparations show that

Quantum physics

Spaced-out electrons

John C. H. Spence

In a stream of photons, the particles tend to bunch together, but electrons in a beam do the opposite. At last, this quantum effect for free electrons — the Hanbury Brown–Twiss anticorrelation — has been seen experimentally.

Like the gentle patter of raindrops, we expect photons, the quanta of sunlight, to arrive at Earth at random intervals, their arrival times distributed in just the same, natural way that customers arrive at a box office to buy tickets for a play. A histogram of the number of people or photons arriving per unit time follows what is known as the Poisson distribution. But in 1909, in the first clear evidence for wave–particle duality, Einstein pointed out¹ that the width of this distribution for sunlight contains both the Poisson contribution of random arrival times, and a second 'wave-noise' contribution, which causes the photons to arrive in bunches.

An experiment performed by Kiesel *et al.*², reported on page 392 of this issue, shows that the same principle applies to a beam of coherent electrons — but with the opposite effect. In contrast to the bunching of photons³, this experiment confirms the theoretical prediction⁴ that a stream of coherent electrons

the capacity for long-term potentiation, the process thought to underlie learning and memory, is reduced under certain conditions. They suggest that this stems from the effect of impaired dystroglycan in the post-synaptic mechanisms of neurotransmission.

Together, the two reports^{3,4} describe a powerful 'full circle' of investigation — from known human disease genes, to recognition of similarities between mouse mutants and human disease, to discovery of common features in the disease processes and identification of a pathway that might be involved. Predicting the outcome of genetic-manipulation experiments in mice, and testing those predictions, completes the circle. The exciting part is that this is only the beginning of understanding the neurobiology of the congenital muscular dystrophies. Yet to come is the unravelling of dystroglycan's role in synapse formation in the brain and in the neuromuscular junction, as well as in neuronal migration and synaptic function.

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will 'anti-bunch', tending to become more equally spaced than the classical Poisson prediction (Fig. 1, overleaf). In the quantum regime, electrons have an innate tendency to avoid each other, thereby demonstrating a fundamental difference in the way light and electrons interfere with themselves.

In the 1950s, the astronomers Robert Hanbury Brown and Richard Twiss devised the famous experimental arrangement known as intensity interferometry to study these effects for photons in the form of light from distant stars⁵. They split the light into two beams using a half-silvered mirror, then compared the arrival times of photons at two separate detectors. Due to bunching, they saw that coincident photon arrivals were more likely than expected by chance. Hanbury Brown and Twiss realized they could use this bunching to infer the angular size of distant stars.

The similar experiment performed by

Kiesel *et al.*² with electrons finds coincident arrivals less probable, which indicates anti-bunching. In fact, although an anti-bunching effect was reported three years ago^{6,7} for electrons in solids, Kiesel and colleagues' experiment is the first to mimic the original of Hanbury Brown and Twiss for a beam of free particles. It is because the effect is so much weaker with electrons that it has taken nearly fifty years to detect it.

Kiesel *et al.* used a field-emission point source of electrons to illuminate two detectors, recording the time between the arrival of electrons at each detector. To observe anti-bunching, the researchers needed to prepare a laser-like group of 'coherent states' for the electrons and attempt to crowd as many electrons into them. By 'coherent', we mean that the waves originate from a common point source, and all vibrate in unison for a period of time, called the coherence time (wave-particle duality tells us that particles can be described as waves). As the product of the coherence time and the energy spread of electrons has a fixed value (equal to Planck's constant, by Heisenberg's uncertainty principle), the authors required an extremely small, bright source of electrons with very small energy spread to maximize the coherence time, and, ideally, a detector capable of responding within this time. Uncertainty results from coherence.

The crowding of particles into a state is measured by degeneracy (or maximum number of particles), δ , which is thus a measure of the strength of anti-bunching and many-particle effects. Electrons belong to a class of particles known as fermions — particles whose spin quantum number has half-integer values. Fermions have the special property that only one particle at most is allowed in a particular quantum state, according to Pauli's famous exclusion principle. In this case, $\delta = 1$. For photons, however, the maximum number of particles per state is unlimited. Photons belong to a different class of particles called bosons, with integer values of spin, and do not follow Pauli's exclusion principle.

Degeneracy is proportional to the source brightness and the cube of the particle wavelength. Although the field-emission source used by Kiesel *et al.* is brighter even than a typical photon source, the synchrotron undulator³, the wavelength of electrons produced is much shorter, so δ is much smaller (about 10^{-4} , compared to 10 for the synchrotron undulator and 10^{12} for photons in a laser). This is what has made the observation of electron anti-bunching so difficult — and the achievement of Kiesel *et al.* so significant. (Incidentally, neutrons, also fermions, have even smaller degeneracies, $\delta = 10^{-10}$, making the observation of neutron anti-bunching a hopeless endeavour.)

As the source brightness is increased (approaching the emission of one electron

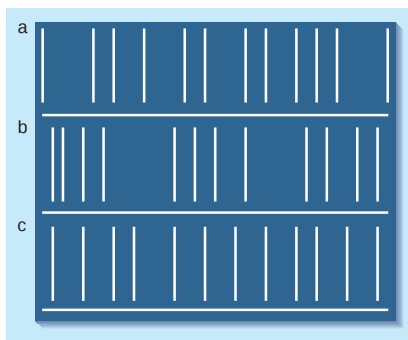


Figure 1 Random and bunched distributions.

a. The vertical lines represent a random sequence, such as the times at which theatre-goers arrive at the box office or photons might arrive at Earth from a distant star.

Mathematically, this distribution was described by Poisson (1781–1840) and bears his name.

b. But in fact the case for photons is not quite so straightforward. Quantum correlations cause the photon arrival times to bunch together — an effect exploited by Hanbury Brown and Twiss to measure the angular size of stars.

c. For electrons, the reverse is true: quantum effects cause free electrons to 'anti-bunch', or spread out. Kiesel *et al.*² have now measured this 'Hanbury Brown–Twiss anticorrelation'.

per interval of coherence time), electrons tend to resist being crowded into the same state. The arrival of an electron at one detector indicates a filled state and so, as two electrons cannot occupy the same state, precludes the arrival of an electron at the other detector for the duration of the coherence time. So the rate of coincidences is

reduced below the Poisson prediction.

A number of experimental considerations complicate this simplified picture. Earlier attempts to perform this experiment have been dogged, for example, by false coincidences caused by capacitive coupling between the closely spaced detectors. To get around this (and other problems), Kiesel *et al.* measured the rate of coincidences for different spatial-coherence conditions, effectively varying the size of the electron source. A large source destroys coherence, and then arrival times follow the classical Poisson distribution. By comparing coincidence rates for different source sizes, the signature of the anti-bunching effect can be isolated.

But the condition for a strong anti-bunching effect — that electrons are emitted from the source at time intervals shorter than the coherence time — cannot be met in Kiesel and colleagues' experiment, as the fastest detectors available are still about a thousand times slower than the longest coherence times possible with field-emission sources. Hence the effect seen is weak, but — at last — it's definitely there. ■

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Medicine

Silencing viruses with RNA

Gordon G. Carmichael

Our cells have a built-in mechanism for 'silencing' genes, called RNA interference. This capability has now been exploited to protect cells in culture dishes from HIV-1 and poliovirus.

Until recently, the two major ways to combat viral infections were vaccines and drugs that are targeted to specific viral enzymes or other viral proteins. Papers on pages 430 and 435 of this issue^{1,2}, and another published earlier this month in *Nature Medicine*³, provide a significant new strategy — one that targets viral genomes and the messenger RNA molecules they encode.

Each of these papers makes use of a revolutionary new technology, RNA interference (RNAi), which prevents the expression of genes by using small RNA molecules, such as 'small interfering RNAs' (siRNAs). This technology in turn takes advantage of the fact that RNAi is a natural biological mechanism for silencing genes in most, if not all,

cells of many living organisms, from plants to insects to mammals⁴. The idea is that RNAi prevents a gene from producing a functional protein by ensuring that the molecular intermediate — the messenger RNA (mRNA) copy of the gene — is destroyed.

How does it work? The key cellular components in RNAi are molecular complexes (the silencing complexes shown in Fig. 1, overleaf) that contain double-stranded siRNAs of 21–23 base pairs in length, associated with a number of proteins, some of which remain to be identified. These complexes recognize their (single-stranded) mRNA targets by matching RNA sequences, and direct the cleavage and destruction of any mRNAs that perfectly match one of the

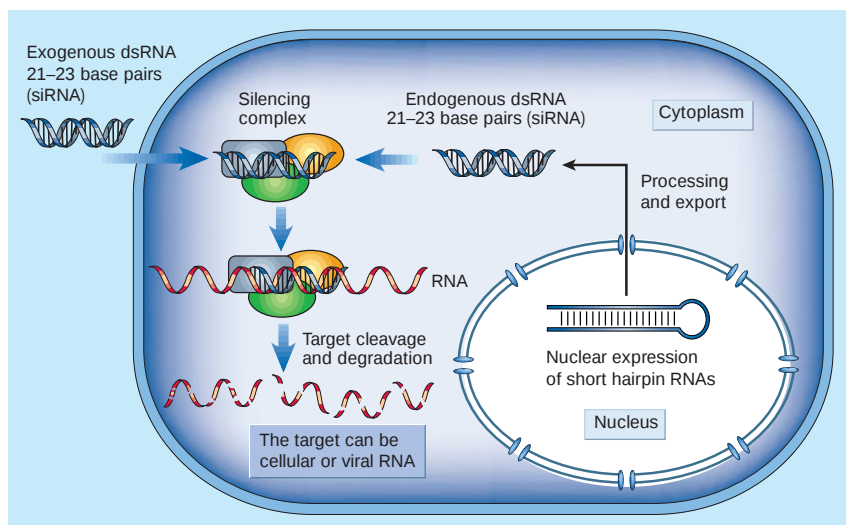


Figure 1 RNA interference — a tool for cells as well as scientists. The diagram shows how RNA interference is thought to occur in mammalian cells. Short double-stranded RNA molecules (dsRNAs) can be introduced into cells from the outside, or are produced within the cell nucleus from longer precursors. These precursors are typically RNAs that can fold back on themselves by base-pairing to form 'hairpin' structures, which are cleaved to generate shorter RNAs, containing 21–23 base pairs, that are then exported to the cytoplasm. In the cytoplasm, the short dsRNAs (called 'small interfering RNAs', or siRNAs) assemble with proteins into silencing complexes. Targets that perfectly match one strand of the dsRNA are cleaved and destroyed. The targets are messenger RNA copies of certain genes, such as those of viruses that have entered the cell. So RNA interference is useful to the cell, and new papers^{1–3,11,12} suggest that it could also be exploited by scientists to fight viral infections.

two siRNA strands^{5–7}. That prevents the mRNAs from being translated into protein. If even a single nucleotide differs between an siRNA and its target, the effect is greatly diminished, or even eliminated entirely. The required length of 21–23 nucleotides is short enough to ensure a low probability that RNAs other than the desired target will be destroyed; siRNAs that find no suitable target appear to remain inert within the cell. Following target recognition and destruction, the silencing complexes may act again on other target molecules.

Silencing complexes can be assembled from siRNAs experimentally supplied from outside the cell, but can also be produced within the cell, using siRNAs processed from precursor RNAs expressed in the cell nucleus. Accordingly, several recent reports^{8–10} have shown that if short 'hairpin' RNAs (which fold back on themselves, generating double-stranded regions) are expressed in the nucleus, they can be trimmed by cellular enzymes to the correct size for RNAi, then assembled into silencing complexes that act outside the nucleus (Fig. 1).

RNAi is useful to cells because it enables them to control gene expression; for example, it might be an ancient defence mechanism that prevents viruses that have infected the cell from producing functional proteins. From a scientist's standpoint, because RNAi relies on nucleotide interactions, it can be tailored to almost any gene — whether cellular or viral — and so can be used to study the function of genes by knocking out their

expression without actually destroying or mutating the genetic material^{1–3,11–14}. And it has appeal for treating viral infections: it offers an attractive combination of exquisite specificity (with no apparent adverse side effects) and the exploitation of an underlying cell pathway that might have evolved naturally for a similar purpose.

But does it actually work as an antiviral weapon? The three new papers^{1–3} suggest that it does, at least in cells in culture dishes. Gitlin and colleagues¹ report that specific siRNAs administered to human cells from the outside, like a drug, can enter them and protect them against infection by the rapidly multiplying poliovirus. Jacque and co-workers², meanwhile, report similar results in their studies with the AIDS virus HIV-1. These authors further demonstrate that if the siRNAs are expressed from inside cells, rather than simply administered to the outside, the cells become largely immune to subsequent HIV-1 infection.

Novina *et al.*³ also show that siRNAs directed against HIV-1 have the potential to be useful treatments. In addition, these authors used siRNAs targeted against the cellular receptor for HIV-1 — CD4 — to reduce the ability of the virus to enter cells. These^{1–3} and other recent studies^{11,12} show that siRNAs can inhibit viral replication at several stages of infection, including the very early stages, when viruses are most vulnerable. Infection can also be blocked by targeting either viral genes or host genes that are involved in the viral life cycle³.

These results are exciting, and suggest that RNAi could be perfectly suited to many antiviral applications. But success is not automatically assured. One important factor is that not all viral RNA sequences are equally accessible to siRNAs: some sequences might be buried within secondary structures or within highly folded regions in target RNAs, whereas others might form tight complexes with proteins that obscure their recognition⁴. Optimal targets must be chosen not only rationally, but also by trial and error.

Another, and quite serious, issue is that viruses are notoriously sloppy in their replication, and produce mutated progeny molecules. Some of these naturally help the viruses escape immune surveillance or inhibition by drugs, but they might also prevent recognition by siRNAs. To overcome this obstacle, one might need to target viral RNA sequences that are conserved and normally invariant between different strains, or to simultaneously target several viral sequences. Finally, the question of how to deliver siRNAs to cells needs to be addressed. They can certainly be delivered efficiently to cells in culture, but methods must be improved before RNAi can be used in animals, let alone patients.

Although antiviral RNAi technology is not yet optimized, the phenomenon does appear to be both general and effective. In 1988, David Baltimore¹⁵ proposed the concept of "intracellular immunization", whereby one would express within cells inhibitory molecules (usually proteins) that can protect those cells from specific viral infections in the future. The promise of intracellular immunization now appears to be closer to reality — although, amazingly, through the use of small RNAs rather than peptides or proteins. ■

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Biogeography

Springboards for springtails

Peter D. Moore

Travel to overseas destinations is possible even for certain tiny land invertebrates. Although they cannot fly, the ocean is not an insuperable barrier because they can survive for longish periods in sea water.

For small, flightless terrestrial organisms, the world is littered with barriers to dispersal. Deserts, oceans and mountain ranges present apparently insuperable limits to migration and spread for animals that are only a few millimetres in size and cannot fly. But from work by S. J. Coulson and colleagues¹, published in *Functional Ecology*, it seems that some soil arthropods can survive long enough in sea water to permit them to travel to distant lands. Assumptions about the causes of biogeographical distribution patterns among such organisms now need to be reconsidered.

Disjunct distributions are relatively common in soil arthropods, as in the case of the springtail *Tetracanthella arctica* (order Collembola), a dark blue, flightless insect, just 1.5 mm long, that lives mainly among mosses on the soil surface and feeds on plant detritus and fungi. It is found in isolated locations through the Arctic, ranging from Spitzbergen, Iceland and Greenland to northern Canada, but also occurs in alpine habitats in the Pyrenees, the Tatra Mountains and the Carpathians². Its isolation in the European mountain sites is best explained by the survival of relict populations after the end of the last glaciation: a presumed extensive distribution in glacial times would have contracted and become fragmented as warmer conditions spread and forest became abundant. But the scattered distribution of this springtail in the Arctic regions could be explained in several ways, including carriage on ice floes, or by larger, more mobile animals (particularly birds), or on drifting chunks of sea-borne turf or driftwood.

On 14 November 1963, an undersea volcanic eruption off the south coast of Iceland led to the formation of a new island, Surtsey, which provided a unique opportunity for observational studies in transoceanic colonization by terrestrial organisms. In 1975, Sturla Fridriksson³ published a summary of this work and recorded that six species of Collembola had reached Surtsey within 10 years of its formation. *Tetracanthella arctica* was not among them, despite being present on some of the Westman Islands only 15 km away. Such small soil organisms as the springtails, however, are evidently capable of quite rapid colonization over distances of several kilometres.

Coulson and his colleagues¹ adopted an experimental approach to the question of dispersal potential. They subjected five

species of Collembola and one mite to a variety of seawater conditions to test whether these terrestrial organisms can survive in the sea, even in the absence of driftwood or other materials. Many springtails have hydrophobic, unwettable cuticles that enable them to survive on water surfaces. Subjecting such species to 16 days on the surface of agitated sea water showed that they could indeed survive this long. Three out of the five species tested (including *T. arctica*) displayed over 80% survival. In a separate experiment, *T. arctica* and the mite *Camisia anomia* were submerged by pushing them below the surface with a paintbrush. They continued to be active, and 75% of the mites survived over a 15-day period. Only 12% of the springtails did so. Yet prolonged survival of even a small proportion of these organisms — which reproduce parthenogenetically, without the need for males — could permit their wide dispersal and subsequent establishment.

The implications for studies of springtail dispersal are considerable. Coulson and Birkemoe⁴ previously demonstrated that some species can survive at a temperature of -22°C for over four years, so the incorpora-

tion of sea-transported springtails into pack ice is entirely possible and could lead to their far-flung travel in the Arctic. Even without the aid of ice, the movement of floating or sunken springtails between land masses such as Norway and Svalbard is quite possible if they can survive for over two weeks in the open ocean.

Distribution patterns of soil invertebrates are of biogeographical interest in a variety of situations. Work in the tropics, for example, has indicated⁵ that termite biogeography and biodiversity might be a reliable indicator of tropical rainforest persistence through the Quaternary, the past 1.6 million years or so. Such hypotheses depend upon the concept of relict, non-mobile populations of an organism, which in turn is underpinned by the assumption of poor dispersal capacity. For termites, the fact that only specialist individuals in a population can start new colonies further limits the mobility of these species, but the revelation of unsuspected methods of travel in a seemingly static animal should always stimulate reflection.

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Sonoluminescence

Inside a micro-reactor

Detlef Lohse

Gas bubbles in a liquid can convert sound energy into light. Detailed measurements of a single bubble show that, in fact, most of the sound energy goes into chemical reactions taking place inside this 'micro-reactor'.

'Single-bubble sonoluminescence' is the remarkable phenomenon that describes how a gas bubble in liquid, exposed to a strong, standing sound wave, collapses and emits light. First observed 12 years ago¹, the basic physics of the process seems to be understood². That there is strong and crucial chemical activity inside the sonoluminescing bubble had already been hypothesized³ and indirectly confirmed^{4,5}. Now Didenko and Suslick⁶ (page 394 of this issue) have performed the first direct measurements of the reaction rates inside an individual bubble as it sonoluminesces. Energy-wise, it seems that a sonoluminescing bubble should be viewed not as a light bulb, but rather as a high-temperature, high-pressure, miniature reactor.

The process of sonoluminescence is shown in Fig. 1, overleaf. First, at low sound pressure, the micrometre-size bubble expands, increasing its volume by a factor of 1,000. When the pressure increases again, the bubble eventually collapses dramatically, shrinking to a radius that corresponds to solid-state densities. The compression drives up the temperature of the gas inside the bubble — through this 'adiabatic' heating the bubble interior is thought to reach around 10,000–20,000 K. Consequently, the gas becomes partly ionized and the recombination of electrons and ions leads to the emission of light.

As the bubble expands, gas dissolved in the liquid enters the bubble. At the point of adiabatic collapse, some of these gases are

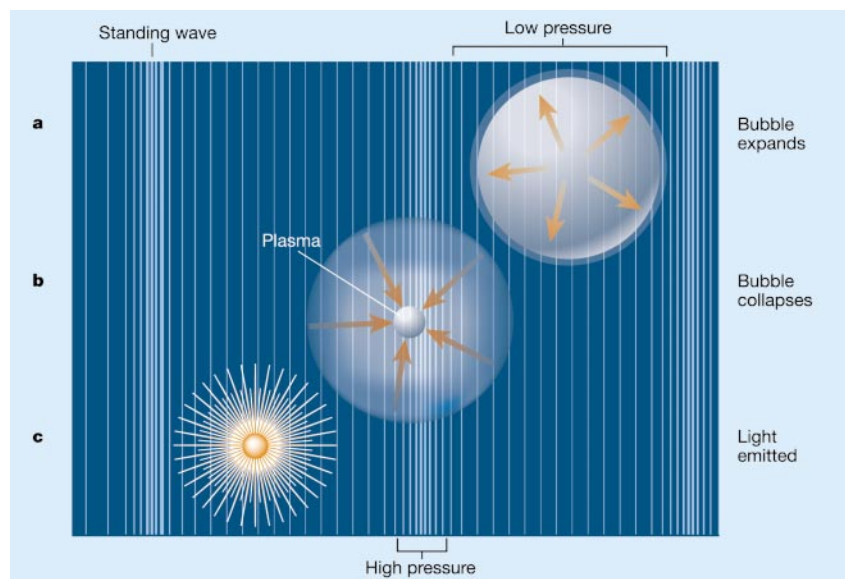


Figure 1 Glowing bubbles: a sound wave in liquid causes sonoluminescence. a, At low pressure, a gas bubble expands dramatically, until b, an increase in sound-wave pressure triggers its collapse. As the temperature inside the bubble soars to over 10,000 K, the gas becomes partly ionized, forming a plasma. Finally, c, recombination of electrons and ions results in light emission. But according to Didenko and Suslick⁶, more energy goes into chemical reactions in the bubble gas than is released as light.

trapped inside the hot bubble and start to react. For example, nitrogen molecules dissociate into nitrogen radicals and then react to form gases such as NH and NO. These highly soluble gases re-dissolve in the surrounding water when the bubble cools down. As the bubble expansion begins again, the next reaction cycle starts. Didenko and Suslick's calculations of the energy budget of sonoluminescence show that the amount of energy going into endothermic chemical reactions inside the bubble is two orders of magnitude higher than that going into light emission.

However, one complication that still remains is that the temperature inside the bubble cannot be measured directly. It has to be deduced either from the bubble dynamics (for example, by Mie scattering^{1,7,8}) or from the properties of the light emitted (spectral information, intensity and widths of the light pulses⁹). Either way, assumptions have to be made, whether in the modelling of the bubble dynamics¹⁰ and the thermodynamics of the heat and mass exchange between the bubble and its surroundings, or in the modelling of the plasma physical processes to predict the observable light properties.

The information obtained in these two ways should obviously be consistent in a viable theory of sonoluminescence. Even then, we can't be certain, as errors arising in the modelling of the bubble interior and light emission could compensate for each other. But Didenko and Suslick's measurements of the chemical reaction rates open up a third experimental window on the process. This extra constraint reduces the freedom in modelling, leading towards further convergence of the models.

It is astounding how many sub-disciplines of physics and chemistry have played a role in disentangling what happens in single-bubble sonoluminescence. They range from acoustics, fluid dynamics, plasma physics, thermodynamics, atomic physics and spectroscopy, to physical and analytical chemistry, chemical kinetics, dynamical-system theory and applied mathematics in general. But nuclear and fusion physics are not on the list: the final conclusion from Didenko and Suslick's results is that it is the chemical reaction rate within the bubble that limits the efficiency of bubble collapse. So 'bubble fusion' — an energy-generating fusion reaction in the high-density, high-temperature interior of the collapsing bubble¹¹ — is most unlikely.

Genetics

Inherit the wheeze

Jeffrey M. Drazen and Scott T. Weiss

A study of families containing asthma sufferers has led to the discovery of a gene that is associated with the disease. The finding brings the biological basis of asthma into sharper focus.

Asthma — a condition that afflicts hundreds of millions of people worldwide — has been recognized by physicians and lay people for more than two millennia. One would think that after all this time, and with so many affected people, we would understand the root causes of the disease. We don't; but we do know a little. Pathologically, asthma is characterized by infiltration of the airways with two specific

Although fusion may be out of reach, there are other uses for sonoluminescent bubbles. The extreme conditions inside the bubble are adjustable through external parameters such as forcing pressure or water temperature, so the bubble can be considered as a controlled high-temperature reaction chamber, offering opportunities to measure reaction rates in extreme temperature and pressure regimes. But understanding single bubbles is not enough. Before this knowledge can be applied to sonochemistry¹² — the enhancement of chemical reactions through ultrasound in a bubbly fluid — a better understanding of bubble–bubble interactions will be needed.

Just as the hydrogen atom was the basic model for larger atoms and molecules, so the single bubble is the simplest building block in the physics of a sound-driven bubbly fluid. With the detailed understanding of the hydrogen atom, atomic physics began to flourish. By analogy, now that there is a basic understanding of single-bubble sonoluminescence and the chemical activity inside the bubble, I expect also a flourishing of cavitation physics.

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requires exposure to certain environmental factors before it is fully expressed.

With all of this understanding, however, we still can't answer the simple question, 'What causes asthma?'. But in a paper on page 426 of this issue, Van Eerdewegh and colleagues¹ describe how they exploited the heritability of asthma to get closer to the solution.

Van Eerdewegh *et al.* have succeeded in identifying a gene that is associated with susceptibility to asthma. The authors initially studied 460 Caucasian families in which there were at least two siblings who were diagnosed as asthmatic by a physician, and who were using medications for their condition. The authors screened the genomes of all the members of these families by using a set of highly variable DNA markers. With this approach — known as linkage analysis — they found that a specific DNA 'signature' on the short arm of chromosome 20 occurred about 1,000 times more often in pairs of affected siblings than would be expected by chance. When Van Eerdewegh *et al.* repeated the analysis using a more restricted definition of asthma (that noted above plus the presence of bronchial hyperresponsiveness), they increased the statistical significance by a factor of ten and narrowed the region of interest to one that contained 23 genes, as defined by the Human Genome Project.

To figure out which of these genes was responsible for the linkage signature, the group identified single nucleotides within the genes that varied from person to person. They then carried out two case-control association analyses, in which they investigated whether a particular sequence variant occurred more frequently in the asthma patients than in nationality-matched controls in US and UK populations. By assessing linkage disequilibrium (the non-random association of genes across the genome) and haplotypes (combinations of single nucleotide variations on one chromosome that tend to be inherited together), Van Eerdewegh *et al.* narrowed the region further and implicated one gene as most commonly associated with asthma. This is the *ADAM33* gene, which encodes a protein-processing enzyme known as a metalloprotease².

Finally, the authors wanted to be sure that the identification of *ADAM33* in the case-control studies was not confounded by different ethnic mixes between cases and controls. So they carried out another family-based study, called the transmission-disequilibrium test, in which the observed frequency of gene variants transmitted from healthy parents to children with asthma was compared to the frequency that would be observed if the gene distribution occurred purely by chance. The results again showed that variations in *ADAM33* are significantly associated with asthma.

Further support for the importance of this gene came from an earlier study³ of the

genetics of bronchial hyperresponsiveness in mice. This study pinpointed a region on mouse chromosome 2 that is close to the region containing a mouse counterpart of *ADAM33*. This striking body of data is strong evidence that *ADAM33* is indeed an asthma-associated gene. It is now up to the community of asthma investigators to determine whether the finding is broadly replicable, and to define the functional implications of this association.

What do we learn from the identification of *ADAM33* as an 'asthma gene'? First, this study would seem to confirm that these genetic techniques can be used to unlock the riddle of complex inherited traits. Second, the results suggest that although asthma and allergy are closely related conditions, they have at least some distinct genetic determinants. In Van Eerdewegh *et al.*'s analysis, linkage was not improved when high levels of the IgE antibody in blood serum — a defining feature of allergy — were included in the definition of asthma. Worldwide, the prevalence of allergy far exceeds that of asthma. So the newly discovered variations in *ADAM33* may be the first genetic clue to what turns the runny nose of a person with allergies into an asthma-related wheezy chest. Third, if the results are replicable, the haplotype defined could be used to identify uniform populations of asthma patients for clinical studies.

Fourth, the most tempting mechanistic interpretation of Van Eerdewegh *et al.*'s data is that the metalloprotease encoded by *ADAM33* has a regulatory role, perhaps in processing proteins that influence fibroblast cells and airway smooth-muscle cells — both of which are known to be activated in asthma. It also seems reasonable to speculate that *ADAM33* could be important in other airway-obstructing diseases, such as chronic obstructive pulmonary disease. But definitive insights await a full functional analysis of normal *ADAM33* and its genetic variants.

We still can't answer the question, 'What causes asthma?'. But we are closer to understanding this complex disease. The study by Van Eerdewegh *et al.*¹ teaches us that there are probably fundamental differences in the airway walls of unaffected people and asthma sufferers. The results broaden the horizons of asthma biologists and physicians beyond a purely immunological interpretation of the disease. This improved understanding is just one benefit derived from the Human Genome Project. ■

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100 YEARS AGO

Alcohol as a motive power has formed an interesting set of experiments in France at the present time, the object being to produce a home-made substitute for petrol which all has to be imported. According to *Heilden's Magazine* for July the results obtained are of a satisfactory nature, both for the heavier and lighter types of cars, and it is stated that passenger cars driven by alcoholic traction have been proved to hold their own against those with petrol as a motive power. The price of alcohol at present is higher, but by the use of beetroot in its manufacture its market value has been greatly reduced. The experiments showed that the amount of alcohol consumed by the engines (which were designed to burn petrol) was 50 per cent. higher than that of petrol, but it is stated that with engines properly constructed to use the new motive power this difference would be greatly reduced... Attention is also directed to the ease with which it can be prepared from potatoes, and consequently, on account of its general utility for heating, lighting, &c., it would seem that an opportunity is open for Ireland to create a most important industry.

From *Nature* 24 July 1902.

50 YEARS AGO

Last season, following the reports that rearing under the bright emitter type of 'infra-red' lamp caused an increased incidence in chicks of a crooked-toe deformity, we reared several hundred chicks from our own stock on solid floors so that we could study the problem at first hand. Despite the fact that we did everything possible to induce the condition... we failed to produce a single case of crooked toe. Towards the end of the season... we were able to obtain some growing birds from an outside source, which had been reared under 'infra-red' and which showed the deformity... Chicks, apparently normal on hatching, from the crooked-toe parents have been reared along with some of our own chicks under the same 'infra-red' lamp, and whereas 100 per cent of the progeny from the crooked-toed parents have developed the deformity, our own chicks have remained quite normal. When second batches of chicks from both sources were reared together, neither the progeny from the crooked-toed parents nor our own stock developed the malformation. It would therefore appear that this particular type of crooked toe is of a hereditary nature.

From *Nature* 26 July 1952.

Magnetic shape-memory effects in a crystal

A surprising new aspect is presented by an antiferromagnetic crystal structure.

Magnetic fields affect the motion of electrons and the orientation of spins in solids, but are thought to have little impact on crystal structure, particularly in compounds with low magnetic susceptibility, such as antiferromagnets. Here we describe an unexpected magnetic effect on crystal shape, in which the direction of the crystal's axes are swapped and the shape changes when a magnetic field is applied; this in turn induces curious memory effects in resistivity and magnetic susceptibility. Ironically, this phenomenon occurs in one of the most well-studied two-dimensional antiferromagnets¹, $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$.

Lightly doped $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ (termed LSCO here) — a prototype layered copper oxide that shows high-temperature superconductivity at moderate Sr doping — has a magnetic susceptibility at room temperature that is as small as that of plastic or paper^{1,2}, so the possibility of magnetic-field-induced structural changes has never been considered in this otherwise well-studied compound.

We have made X-ray measurements that reveal clear changes in the structure of LSCO crystals exposed to strong magnetic fields at room temperature; the magnetic field is somehow capable of swapping the orientation of the crystallographic a and b axes, causing the sample to shrink by about 1% (representing the difference in the two lattice constants) in one direction and to expand along the other.

This crystallographic rearrangement can be viewed in an optical microscope. When LSCO crystals are cooled through the tetragonal–orthorhombic transition, they usually develop a domain structure (known as ‘twins’), where the orthorhombic distortion alters its sign upon crossing the domain boundaries (Fig. 1a). Clear kinks generated by the domain boundaries appear on the initially flat ac/bc crystal faces³ and can easily be seen in the microscope.

We have previously succeeded in detwinning LSCO crystals by using uniaxial pressure, controlling the removal of domain boundaries by optical microscopy^{2,3}. We applied the same technique here to demonstrate the remarkable effect induced by a magnetic field: depending on the field direction, new kinks emerge, move or disappear (Fig. 1b–f) as LSCO crystals try to align their b -axis along the magnetic field by generating and/or moving the domain boundaries.

The specimen shown has been completely detwinned (Fig. 1d, f), as happens after the application of uniaxial pressure,

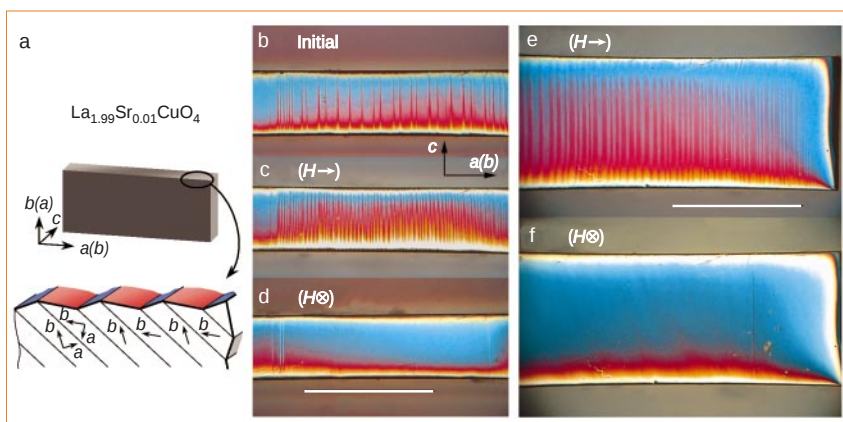


Figure 1 Effect of a strong magnetic field on $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ (LSCO) crystal domain structure. **a**, The orthorhombic-twin pattern, emerging in LSCO below the tetragonal–orthorhombic transition, induces on polished ac/bc faces of crystals a washboard-like modulation that can easily be observed with an optical microscope³. **b–f**, Polarized-light images of the same crystal at room temperature, taken before and after application of the 14-tesla magnetic field along the directions indicated. Vertical stripes in blue and red on the crystal surface correspond to the ac and bc domains, respectively. Initially the crystal was twinned with a larger fraction of ac (blue) domains (**b**). Applying a 14-T magnetic field parallel to the plane of observation at room temperature changed the domain structure, causing the b -axis to align along the field direction and favouring the bc (red) domains (**c**). **e**, Magnified view of the right-hand end of the same crystal. Subsequent application of the magnetic field perpendicular to the plane of observation favoured the ac (blue) domains (**d**, **f**). The magnetic field can remove the twin boundaries and convert the crystal into the single-domain state (**f**). Scale bars, 1 mm (**b–d**) and 0.5 mm (**e**, **f**).

for one of the field directions. For the other field direction, however, perfect alignment is not achieved (Fig. 1c, e), presumably because of some internal crystal strain. Although the crystal structure exhibits such striking agility under the magnetic field, the detwinned state (Fig. 1d, f) is very stable, with no new domain boundaries appearing after storage for six months at room temperature or after being heated to 100 °C.

Manifestations of the rotation of the

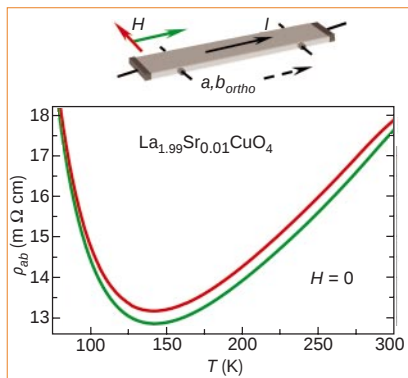


Figure 2 Magnetic-field-induced switching and memory effects in the in-plane resistivity, ρ_{ab} , of LSCO (the crystal is cut along the orthorhombic $a(b)$ axis as shown). At room temperature, the 14-T magnetic field switches the crystallographic b -axis — the direction of better conduction³ — along (transverse to) the current direction, inducing an irreversible decrease (increase) in the resistivity; the plot presents zero-field $\rho_{ab}(T)$ data measured after applying the field $H \perp I$ (red) and $H \parallel I$ (green) at room temperature.

crystal axes are not restricted to changes in sample shape: owing to in-plane anisotropy in physical properties^{2,3}, the swapping of crystallographic axes also induces switching and memory effects in resistivity (Fig. 2) and magnetic susceptibility.

The only other compounds known to exhibit a similar phenomenon are the ferromagnetic shape-memory alloys, where the magnetic field acts on the ferromagnetic moment to produce a torque sufficient to swap the crystallographic axes⁴. Such strong magneto-crystal effects are exotic even among ferromagnets, which interact powerfully with the field. Our findings reveal that the crystal structure of the virtually non-magnetic LSCO can also be manipulated by a magnetic field.

Our findings provide visual evidence of a previously undiscovered strong coupling between the magnetic subsystem and the lattice in copper oxides, offering a missing link in our understanding of these compounds. They also expand the general view of how magnetic fields can affect non-ferromagnetic compounds. As these magnetic shape-memory effects seem to originate from the crystal's magnetic anisotropy² (which is quite widespread in materials), rather than from its strong magnetic moments, they may operate in other antiferromagnetic or paramagnetic compounds as well.

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Pharmacology

Screening inhibitors of anthrax lethal factor

The disease anthrax is caused by lethal factor¹, an enzyme component of the toxin produced by the spore-forming bacterium *Bacillus anthracis*². Here we describe substrate molecules for this factor that offer a means for high-throughput screening of potential inhibitors for use in anthrax treatment³. Our assay should help to answer the urgent call for new and specific therapies⁴ to combat this pathogen after its recent emergence as a terrorist bioweapon.

The clinical presentation and outcome of anthrax in humans depend on the route of entry. Cutaneous anthrax is rarely fatal, whereas systemic anthrax, which follows inhalation of bacterial spores, is more serious. In addition to the metalloprotease lethal factor (LF), the anthrax toxin contains protective antigen, which mediates the entry of LF into macrophages and other cells^{1,5}.

Lethal factor is a proteolytic enzyme

that specifically cleaves signalling proteins of the MAPK-kinase family at their amino termini^{6–9}, targeting a consensus sequence motif⁸ (see supplementary information). We have used this sequence to create *p*-anilide peptide substrates for lethal factor metalloprotease that have favourable kinetic characteristics (see supplementary information). A fluorescent coumarin derivative was also effective as a lethal factor substrate, enabling the activity of minute amounts of lethal factor (5% or less of that detectable with *p*-nitroanilide) to be detected.

With these substrates, lethal factor metalloproteolytic activity can be assayed on plate readers with visible-light or fluorescence detectors (available in most hospital and research laboratories); 1–2 nanograms of lethal factor can be detected in about 200 μ l buffer. Our synthetic substrates should be useful for high-throughput screening of chemical libraries to identify specific inhibitors of lethal factor metalloproteolytic activity.

Conversion of the peptide substrates of metalloproteases into hydroxylamine deri-

vatives generates competitive inhibitors of these enzymes¹⁰. We therefore investigated the inhibition of lethal factor by hydroxylamine derivatives of our peptide substrates *in vitro*, and found that these hydroxamates give nanomolar inhibition constants; the derivative In-2-LF, with a K_i of 1 nM, is the most powerful of these inhibitors.

As anthrax lethal factor acts in the cell cytosol^{2,4}, potential inhibitors must be able to enter cells to be effective. In-1-LF and In-2-LF both include a strongly basic sequence of amino acids with sequences that resemble those of peptides that cross the plasma membrane¹¹. We found that these two peptides inhibit lethal factor's cytotoxicity in the macrophage cell lines RAW264.7 and J774.A1, which are commonly used to assay lethal factor, with In-2-LF again being the more effective (Fig. 1a, b). In-2-LF also inhibits cleavage of MEK-3 (used here as a paradigm of MAPK-kinase cleavage in general; Fig. 1c). Further evidence of the inhibitor's entry into these cells was obtained by using a fluorescein derivative of In-1-LF (results not shown).

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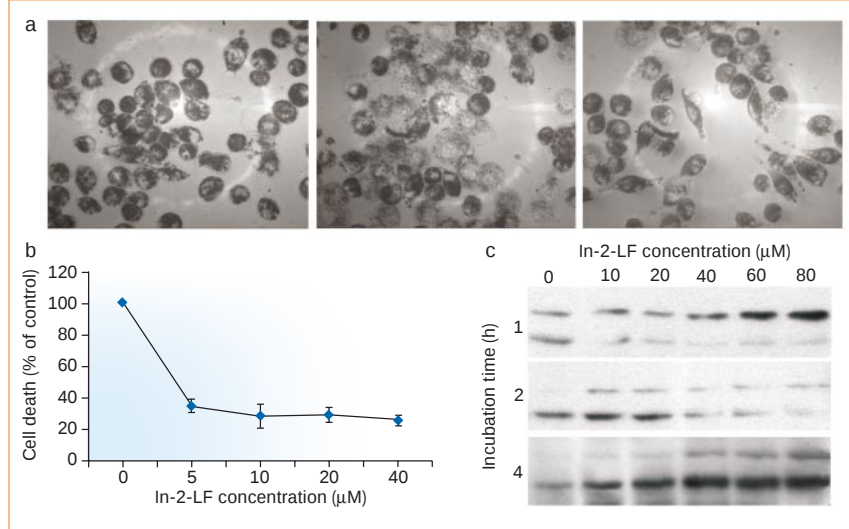


Figure 1 Inhibition by In-2-LF of anthrax lethal factor (LF) in RAW264.7 macrophage cells. **a**, Transmission light micrographs of control cells (left) and of cells treated with anthrax toxin (LeTx, consisting of 200 ng ml⁻¹ LF and 400 ng ml⁻¹ protective antigen (PA)) in the absence (middle) or presence (right) of 40 μ M In-2-LF for 1 h. LeTx at these concentrations kills cells (middle), whereas In-2-LF reduces cell death (right). **b**, Protection of RAW264.7 macrophages by In-2-LF. Cells were plated in 96-well plates at a density of 2×10^4 cells per well in DMEM medium supplemented with heat-inactivated fetal calf serum, 1 d before treatment with LeTx (100 ng ml⁻¹ LF and 400 ng ml⁻¹ PA in serum-free medium); the indicated concentrations of In-2-LF peptide were added 5 min after LeTx. After 2 h, cells were washed with PBS and cell death was assayed with MTS tetrazolium (Promega; control was treated with LeTx only). Results are averages from three independent experiments. **c**, Cleavage of MEK-3b in RAW264.7 cells treated with 800 ng ml⁻¹ LeTx and the indicated concentrations of In-2-LF, as determined by western blotting with anti-MEK3 antibodies (Santa Cruz). Upper and lower bands in each panel are the uncleaved and LF-cleaved forms of MEK-3b, respectively; after 1 or 2 hours of incubation, In-2-LF inhibits the LF-mediated conversion of the uncleaved to the cleaved form in a dose-dependent way (as evaluated by the band ratio), but after 4 hours most MEK-3b has undergone cleavage because In-2-LF acts by competitive inhibition.

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Functional profiling of the *Saccharomyces cerevisiae* genome

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Determining the effect of gene deletion is a fundamental approach to understanding gene function. Conventional genetic screens exhibit biases, and genes contributing to a phenotype are often missed. We systematically constructed a nearly complete collection of gene-deletion mutants (96% of annotated open reading frames, or ORFs) of the yeast *Saccharomyces cerevisiae*. DNA sequences dubbed ‘molecular bar codes’ uniquely identify each strain, enabling their growth to be analysed in parallel and the fitness contribution of each gene to be quantitatively assessed by hybridization to high-density oligonucleotide arrays. We show that previously known and new genes are necessary for optimal growth under six well-studied conditions: high salt, sorbitol, galactose, pH 8, minimal medium and nystatin treatment. Less than 7% of genes that exhibit a significant increase in messenger RNA expression are also required for optimal growth in four of the tested conditions. Our results validate the yeast gene-deletion collection as a valuable resource for functional genomics.

Gene disruption is a fundamental tool of the molecular geneticist and allows the consequence of loss of gene function to be determined. For organisms with facile genetic methods and known genome sequence, it is possible to systematically inactivate each gene^{1–8}. Here we present the construction and initial characterization of the nearly complete set (96% of all annotated ORFs) of gene-disruption mutants in the yeast *Saccharomyces cerevisiae*. This directed approach provides major advantages over classical random mutagenesis and screening. First, the mutant phenotype reflects a complete loss of function of the gene. Second, as a ‘reverse genetic’ approach, the previously laborious task of identifying the gene responsible for the mutant phenotype is accomplished beforehand. Moreover, in contrast to random mutagenesis, where genes often

elude detection even when a large number of mutants are screened, mutant ‘saturation’ of the genome is assured.

Deletion strategy

Each gene was precisely deleted from the start to stop codon (non-inclusive) and replaced by mitotic recombination with the *KanMX* deletion ‘cassette’ shown in Fig. 1 (ref. 9). The *KanMX* gene in each resulting mutant is flanked by two distinct 20-nucleotide sequences that serve as ‘molecular bar codes’ to uniquely identify each deletion mutant (see Methods for details of the design and construction of these sequence tags). Each deletion was verified by several polymerase chain reactions (PCRs), as described in Supplementary Information. In total, we deleted 5,916 genes (96.5% of total

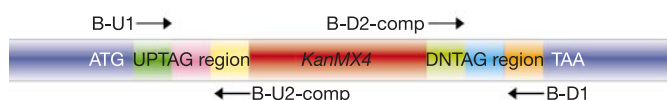


Figure 1 The *KanMX* deletion cassette module. The biotin-labelled, deletion-specific primers (B-U1, B-U2-comp, B-D1 and B-D2-comp; see Methods for structure) are used to amplify the unique UPTAG and DNTAG sequences from genomic preparations generated in the fitness-profiling studies.

attempted; see Methods for a complete tally). Some 18.7% (1,105) of the genes proved essential for growth on rich glucose medium (a list of essential genes is available in Supplementary Information). Only about half of these (57%) were previously known to be essential (M. Cherry, personal communication). Non-essential ORFs are more likely to encode a new protein (17% of the non-essential ORFs and 9% of the essential ORFs encode new proteins); essential genes are more likely to have homologues in other organisms (82% of the essential genes and 67% of the non-essential genes encode proteins that are similar to a protein in another organism), according to the Munich Information Center for Protein Sequences (MIPS) Comprehensive Yeast Genome Database (<http://mips.gsf.de/proj/yeast/CYGD/db>). As expected, few of the essential genes are duplicated within the yeast genome: 8.5% of the non-essential genes, but only 1% of the essential genes have a homologue in the yeast genome.

Whole-genome parallel analysis

The unique sequence tags linked to each gene deletion allow the strains to be analysed in parallel. In each experiment, a mixed culture containing every deletion mutant is grown, samples are collected at several times during growth, and the molecular bar-code tags are amplified from genomic DNA (Fig. 1). The abundance of each deletion strain is then determined by quantifying the associated molecular bar codes by hybridization to an oligonucleo-

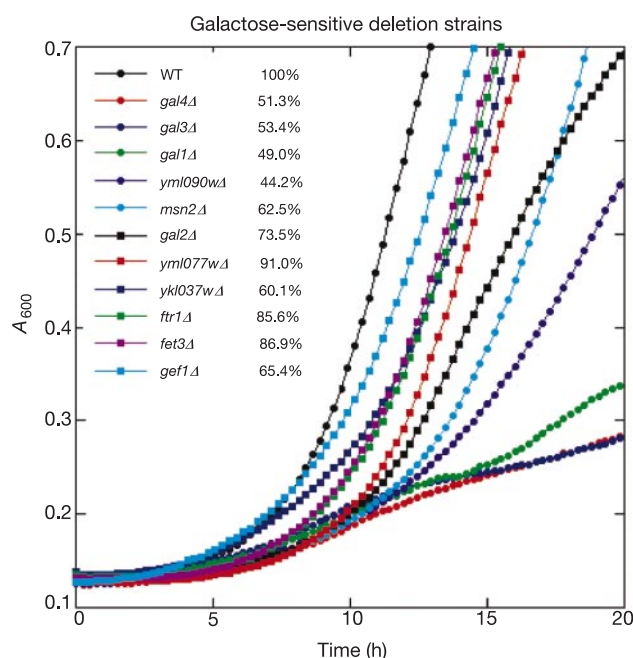


Figure 2 Growth of deletion strains exhibiting reduced fitness in galactose medium. Strains were grown overnight in YPD medium and diluted to 0.1 A_{600} in YPGal the next morning. Growth was in 24-well plates in a 340PC Spectramax spectrophotometer (Molecular Devices). Growth rates were calculated in the exponential phase of the growth curve. Percentage of wild-type (WT) growth is indicated in the legend.

tide array of the complementary bar-code sequences. The more important a gene is for growth, the more rapidly the sequence tags of the corresponding deletion strain diminish in the culture. Thus, all genes required for growth can be identified and ranked in order of their relative contribution to fitness in a single experiment. In the following sections we present results from such fitness profiling of nearly all (96%) of the non-essential yeast genes under several well-studied experimental conditions. Data from these experiments can be found on our website (<http://genomics.lbl.gov/YeastFitnessData>).

Growth in rich medium

About 15% of all viable homozygous deletion strains exhibit a slow growth phenotype in rich medium at 30 °C (for a list of the strains and their functional categories see Supplementary Information Tables S1 and S2). Growth defects cover a continuous range from 12% to 90% of wild-type growth. Genes required for optimal growth under this condition are enriched in the functional categories of protein synthesis and cellular organization (according to the MIPS database¹⁰). Many of these genes encode ribosomal proteins (71.8% versus 59.4% in the whole genome) and proteins involved in mitochondrial function and respiration (28.2% versus 14.8% in the whole genome). Because these proteins are in high demand under such optimal conditions, it is not surprising that they are rate limiting for growth.

Growth in altered environmental conditions

Several adaptive responses to changes in the extracellular milieu have evolved in yeasts. To identify the genes involved in some of these responses, we surveyed all genes for a role in response to amino-acid availability, changes in carbon source, osmolarity/salinity, alkali, and challenge with the antifungal compound nystatin.

The yeast genes involved in amino-acid biosynthesis are well characterized¹¹. We were therefore surprised to discover that 13% of

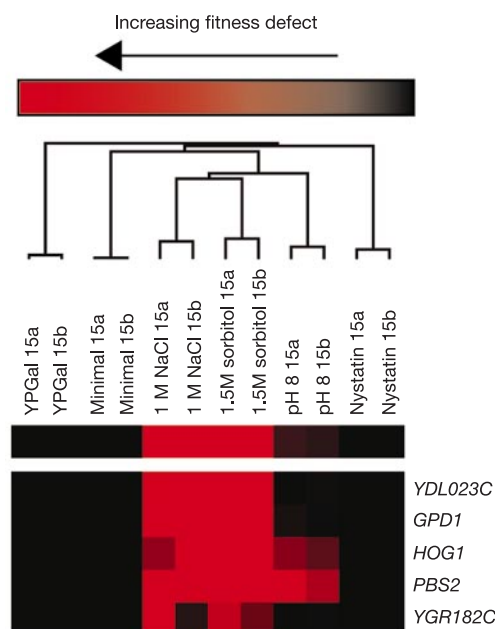


Figure 3 Clustering of genes required for growth in conditions of high osmolarity. Clustered data included fitness defect scores at 15 generations that were greater than 100 in replicate experiments (a, b). Genes were hierarchically clustered across all experiments using the program Cluster²² and viewed in TreeView (<http://rana.lbl.gov/EisenSoftware.htm>). The degree of fitness is represented by a colour bar, with bright red representing strains with the greatest fitness defect. Each row represents a single gene and its behaviour across all 12 experiments.

the genes required for optimal growth in minimal medium (lacking all but the required amino acids) are of unassigned function. When the pool was further grown in media that lacked only threonine, tryptophan or lysine, all genes known for biosynthesis of these amino acids (according to the Kyoto Encyclopedia of Genes and Genomes, <http://www.kegg.com>) were identified. In addition, a new gene—*YJL200c*—that shares similarity with known aconitate hydratases was identified as the component probably responsible for the second step in the lysine biosynthetic pathway (conversion from homocitrate to homo-*cis*-aconitate).

The use of galactose is one of the best-studied pathways in yeast, yet we identified ten genes not previously known to be required for optimal growth on this carbon source: *MSN2*, *FTR1*, *FET3*, *YDR290W*, *ATX1*, *YNL077W*, *YDR269C*, *GEF1*, *YML090w*,

YKL037W (the growth defect of *ykl037wΔ* is probably due to partial disruption of the 5' region of the neighbouring *UGP1* gene, which is required for galactose use). When particular deletion strains were tested individually, they exhibited 44–91% of the wild-type growth (Fig. 2). Thus, fitness profiling can discover genes involved even in previously well-studied pathways.

In wild-type cells, changes in extracellular solute concentration are monitored by two osmotic sensors that independently activate the HOG (high osmolarity glycerol) signal transduction cascade by phosphorylation of Pbs2 (a mitogen-activated protein kinase kinase, or MAPKK). Pbs2 activates Hog1 (a MAPK) that, in turn, leads to the production of Gpd1 (ref. 12). Gpd1 catalyses the rate-limiting step in glycerol production, the process that ultimately returns the cell to homeostasis. As expected, all three of these genes were required for growth in 1.5 M sorbitol and 1 M NaCl (Fig. 3). Three other deletion mutants—*ygr182cΔ*, *gsc1Δ* and *ydl023cΔ*—exhibited significantly reduced fitness in conditions of high osmolarity. Two of these genes were not previously known to be involved in this process. *YGR182c*, a gene expressed upon exposure to 1 M NaCl (ref. 13), clustered with the known responders to osmotic stress discussed above, implying similar function (Fig. 3). In contrast, the *gsc1Δ* mutant, in addition to its sensitivity to high osmolarity, exhibited decreased fitness in minimal and pH 8 media. *GCS1* encodes an ADP-ribosylation factor (ARF) GTPase-activating protein (GAP) protein required for secretion, the absence of which may disable the function of membrane proteins required in these conditions¹⁴. The third unknown ORF required for optimal growth at high osmolarity, *YDL023*, overlaps *GPD1*, suggesting that the phenotype is due to the disruption of the *GPD1* gene and not related to the potential function of *YDL023*.

In addition to causing osmotic stress, 1 M NaCl disturbs ion homeostasis and is ultimately toxic to yeast cells. In response to this insult, cells increase the activity of several of the P-type ATPases, which remove Na⁺ from the cytoplasm through the calcium–calcineurin pathway. We discovered that the calcineurin-related genes *RCN1* and *CNB1* and the protein kinase *HAL5* are critical for growth under ionic stress. Strains deleted for these genes are also sensitive to conditions of high pH, suggesting commonality in the cellular response to these two conditions. Salt-specific targets include the calcineurin-dependent transcription factor Crz1, the ion-transport-related proteins Npr1 and Sat4, components of the Rim1 pathway (Rim101, Rim13), and Sro7. In total, we identified 62 salt-hypersensitive mutants, 47 of which were previously unknown despite two previous genome-wide efforts to identify such genes^{13,15}. It should be noted that deletions of the genes encoding the major ATPases involved in Na⁺ efflux (*ENA1*, *ENA2* and *ENA5*) are not in the yeast knockout collection because their duplicated nature prevented the automated selection of unique primers for making systematic deletions.

In contrast to the pathways regulating growth in response to salt, the cellular response to alkali has not been extensively studied. We identified 128 alkali-hypersensitive mutants, 100 of which are specific to alkali stress, indicating that the cellular response to high external pH is distinct from ionic stress, despite several shared components. Inspection of the genes required for survival in alkaline conditions suggests that proper cell wall maintenance and vesicle transport are required for optimal growth at high pH. These include: components of the Bck1–Slr2 cell wall integrity pathway; members of the Hog1 pathway (Fig. 3), and several members of clathrin-associated protein (AP) complexes. The role of these clathrin-associated proteins in vesicle transport suggests that this process is important for yeast adaptation to high external pH.

Nystatin, one of the oldest and most effective antifungal drugs, causes cell death by binding to membrane ergosterol and creating pores in the plasma membrane. Two of the deletion strains most sensitive to nystatin, *myo5Δ* and *bro1Δ*, are required for cell wall structure and integrity. Consistent with the concept that nystatin

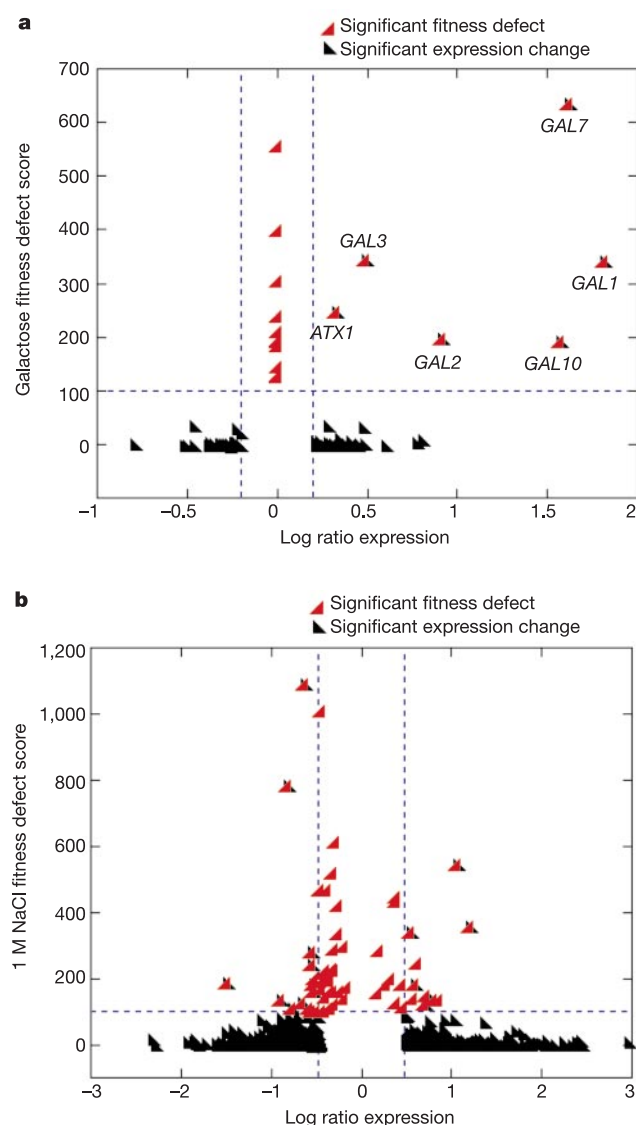


Figure 4 Comparison of expression and fitness profiling data. For clarity, only those genes designated as sensitive by the fitness defect score were plotted. Red triangles represent genes with significant fitness defect scores (above the dashed line) plotted as a function of their corresponding values for log ratio expression: $\log(\text{condition expression}/\text{reference expression})$. Black triangles represent genes with significant log ratio expression (outside the two vertical dashed lines) plotted with their corresponding fitness defect scores. The values of the fitness defects plotted are the minimum score from two experiments. **a**, Galactose. The six points that overlap are significant in both experiments (*GAL1*, *GAL2*, *GAL3*, *GAL7*, *GAL10* and *ATX1*). **b**, 1 M NaCl.

further compromises deletions that are defective in different aspects of membrane integrity, seven of the genes required for optimal growth in 10 μ M nystatin encode either integral or peripheral membrane proteins (*VPS8*, *VPS24*, *VPS28*, *BSD2*, *GIT1*, *MAL11* and *VPS24*). Several other genes required for nystatin resistance are involved in intracellular transport (*STP22*, *YDL100C*, *SRN2*, *SIP3*, *SNF7* and *VPS30*). Eleven genes required for maximal growth in the presence of nystatin are of unknown function, indicating that we still have much to learn about the cellular effects of this compound.

Comparison of fitness and expression profiling

Because both expression profiling and fitness profiling interrogate the whole genome simultaneously, we asked whether a relationship exists between the change in mRNA expression of a gene and its requirement for growth in the same condition. For this comparison, we used previously collected data^{15,16} because strains with the same genetic background as the deletion strains were used in those studies, and expression changes were monitored in four of the same conditions (1 M NaCl, 1.5 M sorbitol, pH 8 and galactose). Our hypothesis was this: if a gene exhibits a significant increase in expression in a given condition, then it should also be required for optimal growth in that condition. We found that in galactose, less than 7% of the genes that exhibited a significant increase in mRNA expression also exhibited a significant decrease in fitness. In the case of pH 8, 1 M NaCl and 1.5 M sorbitol, 3.0%, 0.88% and 0.34%, respectively, of the genes that exhibited a significant increase in mRNA expression also exhibited a significant decrease in fitness (see Supplementary Information). Moreover, many of the genes that exhibited a significant fitness defect did not exhibit a significant change in expression (Fig. 4, see also Supplementary Information). The fact that such a small percentage of the genes that exhibit a significant increase in expression also exhibit a significant fitness defect was unexpected and warrants closer inspection.

Cell shape and size

To identify genes involved in specifying cell shape and size, we visually screened 4,401 of the homozygous diploid deletion mutants by differential interference contrast (DIC) microscopy of fixed cells.

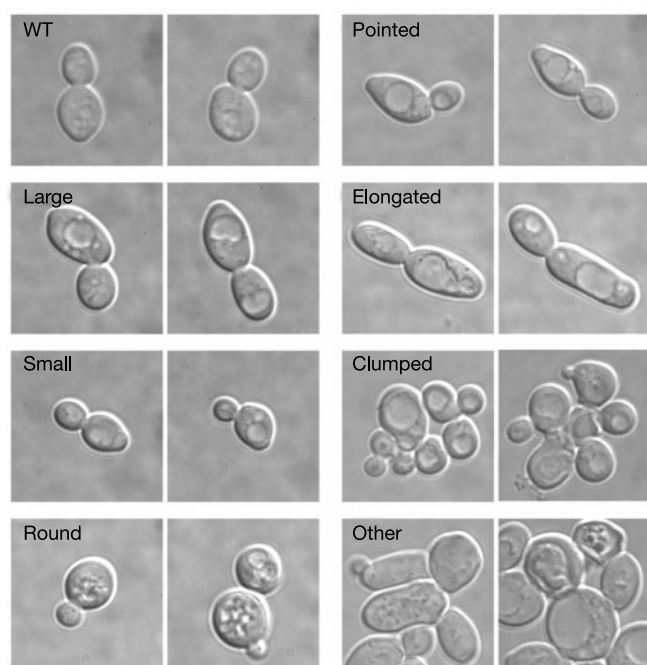


Figure 5 The seven phenotypic categories of deletion mutant morphologies. WT, wild type.

We identified 673 (~15%) deletion strains exhibiting slight to strong morphological alterations from the normal ellipsoid cell shape of wild-type diploid cells. The deletion mutant morphologies were grouped into seven classes: 'elongated', 'round', 'small', 'large', 'pointed', 'clumped' and 'other' (Fig. 5). Mutants with more than three kinds of morphological phenotypes were classified as 'other'. Using the MIPS functional classification system, we found that clumped and elongated strains were enriched for mutations in genes for cell growth, cell division and DNA synthesis (28.9% and 18.9% of clumped and elongated strains, respectively, versus 10.6% for the whole genome). In addition, round strains were enriched for mutations in protein synthesis genes (14.9% versus 3.1% for the whole genome). These latter mutants are also defective in bud site selection, consistent with the hypothesis that apical growth is important for bud site selection¹⁷. A summary of the number of different deletion mutants in each phenotypic category is presented in Supplementary Information along with a detailed list of these strains.

Discussion

The sequence tags that uniquely identify each gene disruption enable functional analysis of the deletion collection on an unprecedented scale. Using this method, we scored the fitness of each homozygous deletion strain (and therefore the requirement for its gene product) under six different conditions. These results confirmed genes known to be required for the different stress conditions, but, more importantly, revealed new genes involved in these processes. Although we did not, to our knowledge, miss any of the genes involved in these pathways, we do expect a small percentage of false negatives in cases where the sequence tags of a strain hybridize poorly to the oligonucleotide array. In some cases, fitness profiling allowed the identification of the gene in a gene pair or family primarily required for a particular process. For example, the *gpd1Δ*, but not the *gpd2Δ*, deletion mutant exhibited reduced fitness in 1.5 M sorbitol. That we uncovered previously unknown genes even in such well-studied pathways as galactose use and amino-acid biosynthesis suggests that we have achieved a higher level of saturation genetics, avoiding the biases known to exist using conventional screens^{3,18}.

We observed little overlap of genes identified both as significant by fitness profiling and as significantly upregulated by gene expression profiling in conditions of 1 M NaCl, 1.5 M sorbitol, pH 8 and galactose. It is easy to imagine why some genes required for growth under a particular condition do not exhibit a change in expression in that condition, because the response to the change in condition may operate post-transcriptionally. The converse situation—a gene that exhibits a significant increase in expression but is not required for growth—is quite surprising, and more difficult to comprehend. It is possible that under stress conditions, multiple gene products are expressed, only a small fraction of which are essential for adaptation to the specific condition in question. Some of these differences might also be ascribed to the highly duplicated nature of the yeast genome. Whatever the cause, fitness profiling may help to identify the subset of genes identified in expression profiling that are required to be expressed to adapt to the condition in question. □

Methods

Deletion strains, primer choice and synthesis

For details of deletion strain construction and primer choice and synthesis, see Supplementary Information. For strain availability, see our website (<http://www-deletion.stanford.edu>).

Selection of genes to be deleted

The initial annotated ORF list obtained from the *Saccharomyces* genome database (SGD, <http://genome-www.stanford.edu/Saccharomyces>) included 6,227 unique ORFs, but was pared down to 6,131 ORFs after removal of ORFs that are not unique owing to gene duplication or regions of high sequence similarity. Of these, we generated four yeast gene

knockout (YKO) collections: 4,815 *MATa* and 4,803 *MATα* haploid deletion mutants (independently generated) deleted for non-essential genes, 4,757 homozygous diploid deletion mutants missing non-essential genes, and 5,916 heterozygous diploids (including essential and non-essential genes). We failed to delete 215 genes for unknown reasons; about 62% of these are questionable ORFs that have no known biological function. The list of ORFs not deleted in the YKO collection can be found in Supplementary Information.

Media and growth conditions

YPD (yeast extract, peptone, dextrose) and synthetic minimal media were prepared as described^{19,20}. Minimal drop-in medium included histidine, uracil and leucine, which are required for growth of the deletion strains. We added 1 M NaCl and 1.5 M sorbitol as supplements to YPD before autoclaving. YPGal medium is equivalent to YPD medium except that 2% galactose is substituted for 2% dextrose. We made pH 8 medium by titrating YPD with 1 M Tris-HCl buffer at pH 9.6 (~10 ml l⁻¹).

Deletion pool construction and growth

Pools of the deletion mutants were prepared as follows: batches of 96 deletion strains were applied in patches to YPD plates and grown for 3 days at 30 °C. Approximately five absorbance units at 600 nm (*A*₆₀₀) of cells of each strain were collected from solid medium with wooden toothpicks and added to 25 ml of YPD plus 15% glycerol. The subpools were stored in 1-ml aliquots at -80 °C. To construct the whole genome pool, subpools were thawed and mixed together such that the average *A*₆₀₀ per strain was equivalent and aliquots were stored at -80 °C. In each experiment, ~6 × 10⁸ cells from a freshly thawed pool aliquot of homozygous deletion mutants (~10³ cells per strain per culture) were inoculated in YPD and grown overnight to allow about ten generations of recovery from storage at -80 °C. Cells were then diluted into 50 ml of the appropriate pre-warmed fresh media and grown at 30 °C with shaking at ~250–300 r.p.m. in 250-ml flasks. To minimize sampling errors while maintaining logarithmic growth, cultures were batch diluted as necessary to not less than ~10³ cells per strain. We collected 2 *A*₆₀₀ of cells from the cultures at 5 and 15 generations after the recovery period and froze them at -20 °C for subsequent preparation of genomic DNA.

Genomic DNA preparation, PCR and chip hybridization

DNA from 2 *A*₆₀₀ of cells was prepared after lysing the cells either with glass beads²¹ or enzymatically using a Qiagen DNeasy kit. The UPTAG and DNTAG molecular bar codes were amplified from ~0.2 µg of genomic DNA in two separate reactions. The UPTAG amplification used primers B-U1 (5'-biotin-GATGTCCACGAGGTCTCT) and B-U2-comp (5'-biotin-GTCGACCTGCAGCGTACG); the DNTAG amplification used B-D1 (5'-biotin-CGGTGTCTCGTCTCGTAG) and B-D2-comp (5'-biotin-CGAGCTCGAATTCATCG) (Fig. 1). Amplified UPTAG and DNTAG sequences were combined and used to probe high-density oligonucleotide arrays (Affymetrix Tag3 array) in 150 µl of 1X hybridization buffer (100 mM MES, 1 M Na⁺, 20 mM EDTA, 0.01% Tween 20 and 1X Denhardt's solution) containing 1 µM of U1, U2, D1 and D2 oligonucleotides and their complements, and 0.6 fM of B213 control oligonucleotide. Samples were boiled for 2 min, chilled on ice for 2 min, and hybridized at 42 °C for 16 h. Washing, staining and scanning were performed as previously described².

Data analysis

For a description of the data analysis and for access to complete data sets, see Supplementary Information and our website (<http://genomics.lbl.gov/YeastFitnessData>).

Screening deletion mutants for cell morphology

For a description of the cell morphology screens see Supplementary Information.

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Supplementary Information accompanies the paper on *Nature's* website (<http://www.nature.com/nature>) and is also available on the authors' websites (<http://yeastdeletion.stanford.edu> and <http://genomics.lbl.gov/YeastFitnessData>).

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Competing interests statement

The authors declare that they have no competing financial interests.

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Observation of Hanbury Brown–Twiss anticorrelations for free electrons

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Fluctuations in the counting rate of photons originating from uncorrelated point sources become, within the coherently illuminated area, slightly enhanced compared to a random sequence of classical particles. This phenomenon, known in astronomy as the Hanbury Brown–Twiss effect^{1–5}, is a consequence of quantum interference between two indistinguishable photons and Bose–Einstein statistics⁶. The latter require that the composite bosonic wavefunction is a symmetric superposition of the two possible paths. For fermions, the corresponding two-particle wavefunction is antisymmetric: this excludes overlapping wave trains, which are forbidden by the Pauli exclusion principle. Here we use an electron field emitter to coherently illuminate two detectors, and find anticorrelations in the arrival times of the free electrons. The particle beam has low degeneracy (about 10^{-4} electrons per cell in phase space); as such, our experiment represents the fermionic twin of the Hanbury Brown–Twiss effect for photons.

Correlations between successive detections in beams of bosons or fermions are observed even if the emission of the two particles cannot physically influence one another because, for example, the emission sites have a space-like separation. In such a radiation field, correlation builds up in the process of propagation. At points sufficiently far from the sources the field becomes highly correlated. This observation of Hanbury Brown and Twiss in 1956 seemed so strange to many physicists that they declared it physically absurd⁷, although the van Cittert Zernike theorem of partial coherence, which expresses the field correlations (coherence) at two points in an optical field emerging from incoherent planar sources, was formulated nearly two decades before. (See ref. 8 for discussions on this controversy.)

In essence, Hanbury Brown and Twiss have shown that in addition to observing interference fringes, measuring field correlations (second-order coherence) provides another sometimes more convenient and powerful method for measuring the absolute value of the degree of coherence. For example, with their stellar intensity interferometer, Hanbury Brown and Twiss were able to measure the diameter of Sirius, undisturbed by fluctuations of the refractive index of the atmosphere⁹. The reason why (anti)correlations between free photons (electrons) in coherent beams are so difficult to observe, and why the fundamentally different behaviour of free bosons and fermions does not become obvious in light and electron optics—in spite of the extreme difference between Bose–Einstein and Fermi–Dirac statistics—is that the occupation numbers (degeneracy) in phase space are very low. Only recently, with the advent of high-brightness field electron emitters with degeneracies reaching 10^{-4} , has an experiment for free fermions become feasible^{10,11}. The first fermionic Hanbury Brown and Twiss experiments in semiconductor devices, where highly degenerate beams of electrons are conveniently available, were performed in 1999 (refs 12, 13).

The coincidence method, in which two detectors are coherently illuminated by an electron field emitter, chosen in the present ‘antibunching’ experiment is an alternative to the correlation procedure of Hanbury Brown and Twiss for gathering information about correlations in a stream of particles¹⁴. According to the Pauli principle, no two electrons having a certain spin direction are allowed to be in the same quantum state, that is, they cannot arrive at both detectors simultaneously. In other words, if our detectors

had a time resolution corresponding to the coherence time T_c which is of the order of 10^{-14} s, no coincidences would be observed. (The fluctuations in orthogonal polarizations or spin directions are independent. Therefore, because the experiment is performed with unpolarized electrons, suppression of fluctuations is only 50% compared to a polarized beam.)

The time resolution of our fast coincidence counter of $T_r = 26$ ps was about three orders of magnitude less than the coherence time. For incoherent illumination of the detectors, the electrons behave like classical particles. Owing to the insufficient time resolution a certain random coincidence rate is observed. When we change the illumination from incoherent to coherent we expect a reduction of the random coincidences (by a factor T_c/T_r of about 10^{-3} in the present experiment) because no second electron is allowed to arrive within 10^{-14} s after the arrival of the first electron. This reduction is our signature of antibunching.

Our experimental set-up corresponds to the stellar interferometer of Hanbury Brown and Twiss. The tiny effective virtual source of an electron field emitter illuminates, by means of magnifying quadrupoles, two small collectors (Fig. 1, right hand side). With

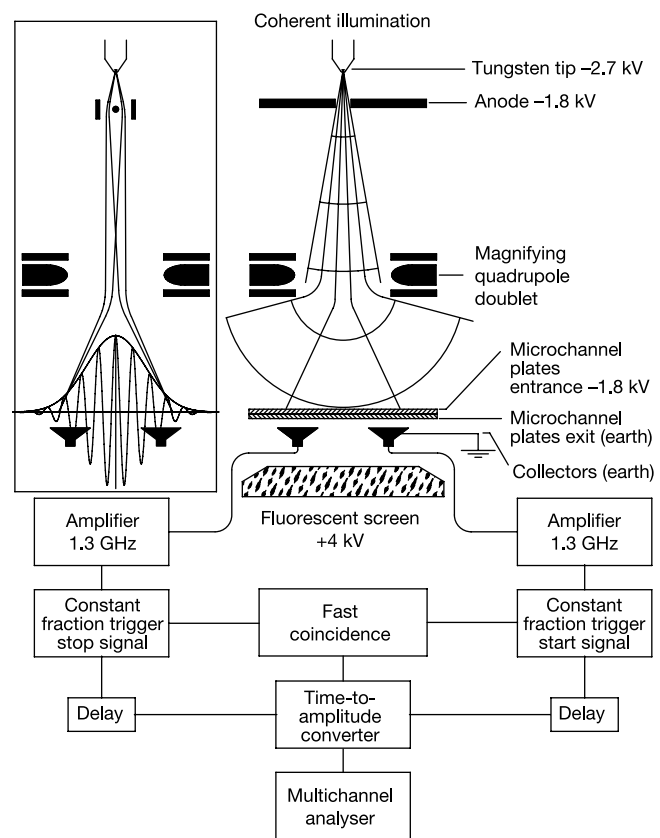


Figure 1 Electron optical set-up (top) and fast coincidence electronics (bottom) to measure electron anticorrelations. The quadrupoles produce an elliptically shaped beam of coherent electrons (schematically shown in the pictograms of Fig. 3). For geometrical reasons, fewer coherent electrons miss the collectors than for isotropic magnification. This greatly reduces the measuring time T_M . The parts of the spherical cones emerging from the cathode represent single coherence volumina. Between the electron source and the quadrupole a biprism (inset) is inserted temporarily to check the coherence of illumination of the collectors. The very short electron avalanches (rise time and width of about 0.5 ns) leaving the channel plates are transferred coaxially from the collectors via microwave amplifiers (bandwidth 1.3 GHz) to modified constant fraction trigger modules that extract timing signals with low variance of transit time resulting in a very good time resolution. A fast coincidence circuit preselects events within a time window of ± 3 ns and opens the gate of a time-to-amplitude converter. The time spectra with a resolution of 26 ps are accumulated by a multichannel analyser. Capacitive crosstalk between the collectors was well below 1% and did not cause spurious coincidences.

Table 1 Summary of results

	Illumination		
	Partially coherent	Coherent	Partially coherent
$S = \Delta N$	4.567	16.942	8.292
$S_{rel} (\times 10^{-3})$	0.61	1.26	0.34
Signal-to-noise ratio	2.2	3.0	1.4
T_M (min)	1,402	1,690	4,531
$T_{M,eff}$ (min)	1,402	1,642	4,450

S and S_{rel} are the absolute and relative coincidence reduction, respectively, T_M is the total measuring time and $T_{M,eff}$ is the effective measuring time after subtraction of times of unstable emission of the field emitter.

increasing angular magnification, the effective lateral distance of the collectors decreases and their illumination changes from incoherent to totally coherent. In turn, a continuous increase of anticorrelations of arrival times of the electrons at the collectors is expected.

The collectors (Fig. 1), 4 mm in diameter with an impedance of 50Ω , inserted between the exit of the two cascaded microchannel plates and the fluorescent screen, collect the electron avalanches initiated by single electrons. Into the actual electron optical set-up—constructed according to the principles used in our miniaturized electron interferometer¹⁵—an electron biprism (inset to Fig. 1) is integrated. This allows us to examine the state of coherence of illumination of the collectors—at least in the direction perpendicular to the fringes—by observing the overlap of the fringes with the shadows cast by the collectors on the fluorescent screen. Thus we determined the magnification factors that are necessary for coherent, partially coherent and incoherent illumination of the collectors. The antibunching experiments were performed without a biprism in the beam path at these predetermined magnifications.

Field emission of electrons is a poissonian process and so—for incoherent illumination—the probability of arrival of an electron after an elapsed time $\tau = t_1 - t_0$ is given by $P(\tau) = e^{-\bar{n}\tau}$ where $\bar{n}$ is the average counting rate. That is, given a start signal at time t_0 , the probability for the next stop signal at t_1 depends exponentially on the counting rate $\bar{n}_{stop}$ in the stop channel.

In semilogarithmic representation, this probability is represented by a straight line with a slope given by $-\bar{n}_{stop}$ (Fig. 2). Correspondingly, stop signals arriving before the start signal are displayed on the

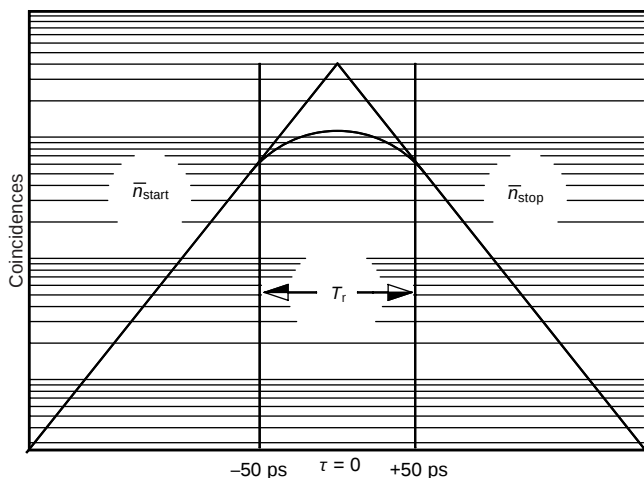


Figure 2 Schematic time spectra expected for poissonian processes at infinite time resolution (straight lines) and an antibunched beam at finite resolving time in semilogarithmic representation. An additional delay of 3 ns in the stop channel of the time-to-amplitude converter causes (see Fig. 1) the arrival time zero to show up in the centre of the time spectra ($\tau = 0$). Stop signals arriving before the start signal are displayed on the negative time axis. Within the resolution time T_r the anticorrelations in a coherent beam of fermions are responsible for the decrease of the random coincidence rate. Quantitatively, the area enclosed between the straight lines and the antibunched curve below corresponds to a loss in the number of coincidences.

negative time axis with a slope of $\bar{n}_{start}$ (Fig. 2). The peak of the experimental time spectrum is rounded within the resolution time window T_r of the time-to-amplitude converter.

The cold (100)-oriented tungsten field emitter that we used has an extraction voltage of 900 V, a total current of $1.5 \mu\text{A}$, an energy full-width at half-maximum ΔE_{FWHM} of 0.3 eV that corresponds to a standard deviation ΔE of 0.13 eV (calculated under the assumption of a gaussian energy distribution), a virtual source diameter of about 36 nm, a brightness of $4.4 \times 10^7 \text{ A cm}^{-2} \text{ sr}^{-1}$, a coherence time T_c of $3.25 \times 10^{-14} \text{ s}$, a coherent particle current of $4.7 \times 10^9 \text{ s}^{-1}$, and a degeneracy of 1.6×10^{-4} .

In spite of a vacuum of 10^{-10} mbar , and a constant total emission current during the data accumulation times T_M (see Table 1), the counting rates of the detectors sometimes increased by more than a factor of two or fell below half of the desired value. In these cases a

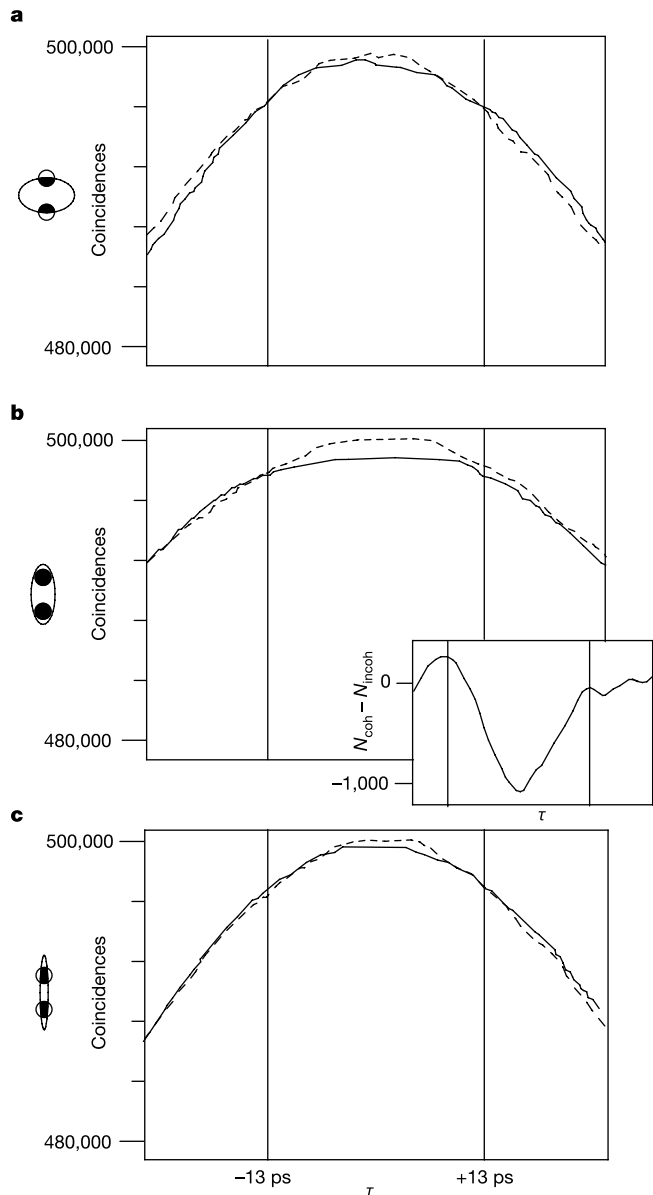


Figure 3 Antibunching as a function of coherence of illumination of the collectors. The coincidences per channel (channel width 0.99 ps) for incoherent illumination (dashed lines) is compared to that for partially coherent (a), coherent (b), and again partially coherent illumination (c) (full lines). In the inset to b the difference between fully coherent illumination, $N_{coh} - N_{incoh}$, is given. In the pictograms the ellipses represent the coherently illuminated areas, the circles the collector areas. The coherently illuminated parts of the collectors are marked in black.

reduction or an increase in coherence of illumination of the collectors took place. Therefore, time spectra with counting rates outside these limits were discarded.

To prove antibunching, a total of four spectra were accumulated for about 30 h each. The first spectrum was for incoherent illumination of the detectors; the next three spectra were, respectively, for partially coherent, totally coherent and then again for partially coherent illumination. The evaluation procedure of the experimental time spectra consisted of smoothing and normalizing the spectra, followed by visually superimposing the incoherent with the coherent and partially coherent spectra, respectively. The results are summarized in Fig. 3 and Table 1. The antibunching signal S , that is, the missing coincidences ΔN in the coherent and partially coherent spectra versus the incoherent spectrum, becomes visible as a flattening of the peak in Fig. 3 within the time resolution window of ± 13 ps. The measured relative reduction in coincidences amounts to $S_{\text{rel}} = 1.26 \times 10^{-3}$ with a signal-to-noise ratio of 3. This agrees with the theoretically expected value calculated from the characteristic features of our cold tungsten field emitter. As expected, the reduction in coincidence rate and the signal-to-noise ratio are smaller for partially coherent illumination.

Antibunching—in general, interference between two particles (second order coherence)—has now been observed for massive free particles. This experimental technique opens a gateway to new fundamental tests of quantum mechanics and statistics: for example, observation of quantum statistics on interference phenomena, and experimental tests of interaction of fields and potentials with charged two-fermion systems^{16–19}. □

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The energy efficiency of formation of photons, radicals and ions during single-bubble cavitation

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It is extremely difficult to perform a quantitative analysis of the chemistry^{1,2} associated with multibubble cavitation: unknown parameters include the number of active bubbles, the acoustic pressure acting on each bubble and the bubble size distribution. Single-bubble sonoluminescence^{3–7} (characterized by the emission of picosecond flashes of light) results from nonlinear pulsations of an isolated vapour-gas bubble in an acoustic field. Although the latter offers a much simpler environment in which to study the chemical activity of cavitation, quantitative measurements have been hindered by the tiny amount of reacting gas within a single bubble (typically $<10^{-13}$ mol). Here we demonstrate the existence of chemical reactions within a single cavitating bubble, and quantify the sources of energy dissipation during bubble collapse. We measure the yields of nitrite ions, hydroxyl radicals and photons. The energy efficiency of hydroxyl radical formation is comparable to that in multibubble cavitation, but the energy efficiency of light emission is much higher. The observed rate of nitrite formation is in good agreement with the calculated diffusion rate of nitrogen into the bubble. We note that the temperatures attained in single-bubble cavitation in liquids with significant vapour pressures will be substantially limited by the endothermic chemical reactions of the polyatomic species inside the collapsing bubble.

Single-bubble sonoluminescence (SBSL)^{3,4} has attracted much attention owing to both the apparent simplicity of its creation and the complexity of processes occurring during bubble pulsation. A single bubble of gas can be trapped in partially degassed liquid by a standing acoustic wave and driven into highly nonlinear pulsations. At appropriate acoustic intensities, the bubble can emit short (~ 50 –

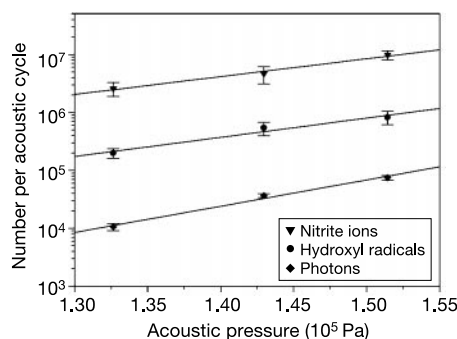


Figure 1 The yields of nitrite ions, hydroxyl radicals, and photons from a single cavitating bubble at 52 kHz in a 15-ml glass cell at 3 °C. The SBSL apparatus is described elsewhere²⁶. Solutions of 10^{-3} M disodium terephthalate in high-purity water were used to measure the yields of hydroxyl radicals. Liquid was degassed and equilibrated with an air pressure of 150 torr. The fluorescence of the product, 2-hydroxyterephthalate, was measured at 425 nm (excitation: 315 nm)²⁷. 2-hydroxyterephthalic acid was synthesized²⁸ and used as a standard after calibration for the OH[•] trapping efficiency²⁹. The yields of NO₂[−] were measured from the fluorescence at 405 nm (excitation: 365 nm) of the product of reaction of NO₂[−] with 2,3-diaminonaphthalene³⁰. The acoustic pressure at the position of the bubble was measured with a needle hydrophone (DAPCO), which was calibrated against a Bruel and Kjaer 8103 hydrophone³¹.

500 ps) flashes^{6,7} of light with clock-like regularity⁵. The featureless spectra of SBSL from 200 to 800 nm with the increasing intensity toward the ultraviolet^{6,8} suggested the existence of extraordinary temperatures inside the bubble, with black-body effective temperatures⁸ as high as $\sim 20,000$ K. Ionization of the bubble contents would also occur at these temperatures, so SBSL is now generally believed to be due, at least in part, to black-body radiation, bremsstrahlung and ion–electron recombination processes^{8–10}.

Before the discovery of SBSL, research on sonoluminescence and sonochemistry was limited to studies of clouds of cavitating bubbles formed in high-intensity ultrasound field^{1,2}. Using sensitive fluorescent analyses, we now report the yields of hydroxyl radicals ($\text{OH}^\bullet$) and nitrite ions (NO_2^-) from a single cavitation bubble. This allowed us to determine energetic characteristics of sonochemical activity of acoustic cavitation. We also present quantitative comparisons between single-bubble sonochemical rates and sonoluminescence intensity.

A bubble pulsating in water containing dissolved air is thought to contain primarily argon, because the N_2 , O_2 and H_2O that diffuse into the bubble during expansion should burn off to form soluble products during bubble compression: the “dissociation hypothesis”¹¹. The expected initial products of chemical reactions inside the bubble include $\text{OH}^\bullet$ and nitrogen oxides (NO_x). $\text{OH}^\bullet$ will react with organic compounds in the water or dimerize to H_2O_2 , and NO_x will react with water giving nitrite and nitrate ions.

The formation of $\text{OH}^\bullet$ in multibubble sonochemistry of water was predicted more than 50 years ago and measured quantitatively using EPR spin-trap methods¹². The formation of nitrite and nitrate ions in multibubble sonochemistry has also been measured, and the yield of NO_3^- is much lower than NO_2^- (ref. 13).

We have now measured the yields of $\text{OH}^\bullet$ and NO_2^- from a single cavitating bubble in water at 28 and 52 kHz, and at 3 °C and 22 °C. Most experiments were conducted at 52 kHz, as the smaller cell volume gives a higher concentration of products. Spectra of SBSL were collected under identical conditions, and a direct correlation between the yields of photons and chemical products was observed. In order to quantify gas diffusion processes, we also measured the size of the cavitating bubble throughout its cycle by a direct stroboscopic method¹⁴. The yields of photons, $\text{OH}^\bullet$, and NO_2^- increase with increasing acoustic intensity, and the yields of $\text{OH}^\bullet$ and NO_2^- are substantially higher than the number of photons emitted (Fig. 1).

Our data allow a meaningful estimate of the yield of radicals and ions per joule of absorbed energy (that is, a *G* value) for acoustic cavitation. From Table 1, the *G* value for $\text{OH}^\bullet$ formation during single bubble cavitation is $1 \times 10^{-10} \text{ mol J}^{-1}$ comparable to $3 \times 10^{-10} \text{ mol J}^{-1}$ for multibubble sonochemistry¹⁵, but much less than typical radiolysis *G* values of $3 \times 10^{-7} \text{ mol J}^{-1}$. Previous reports of *G* values for multibubble sonochemistry¹⁵ are difficult to interpret because the absorbed acoustic energy is distributed among all bubbles in the cloud to varying degrees, and not

exclusively to the few strongly cavitating bubbles that are chemically active at any one time.

The potential energy possessed by a single bubble¹⁶ at its maximum size, R_{max} , is the upper limit of the energy available for the formation of radicals and ions. During bubble compression, the potential energy of the bubble is converted into mechanical energy (both bubble/liquid wall motion and shock waves into the liquid), heat, chemical reactions and light emission. The data in Table 1 on the yields of photons, radicals and ions can be used to calculate the energy balance of a collapsing bubble. From the endothermicity of the sonochemical reactions, we estimate that $\sim 1 \times 10^{-4}$ of potential energy of the bubble goes to the formation of radicals and ions that escape the collapsing bubble, and that the energy of the photons is another factor of 100 less.

This represents, however, only the lower limit of chemical reactions within the bubble. Substantial recombination of radicals or ions must occur inside the collapsing bubble and will be converted to heat¹⁷. For a 30- μm -radius bubble containing 0.01 bar of water vapour, there are $\sim 2.6 \times 10^{10}$ water molecules present; to dissociate all of these to hydrogen and oxygen atoms would require $\sim 2.5 \times 10^{11} \text{ eV}$, that is, several times the total potential energy of the bubble. It is likely, therefore, that sonochemical reactions within the bubble are the primary limiter of the efficiency of the bubble collapse.

Spectra of SBSL were collected under identical conditions to those used for the chemical analyses. As shown in Figs 2 and 3, the spectral distribution of SBSL does not change with an increase in acoustic intensity or of frequency. However, the spectra of sonoluminescence are dependent on the water temperature. This effect has been previously reported for different gases dissolved in water^{4,18}. The ultraviolet component of SBSL is significantly less intense at 22 °C even though the overall SBSL intensity is the same or even higher at 22 °C compared to 3 °C (Figs 2 and 3). It is worth noting that our measured energy efficiency of photon production by SBSL is $\sim 10^4$ higher than that estimated grossly for multibubble sonoluminescence¹⁹. In SBSL, substantial emission of deep-ultraviolet photons that are absorbed by the liquid is likely to occur; this means that our estimate of the energy efficiency of photon production is only a lower limit.

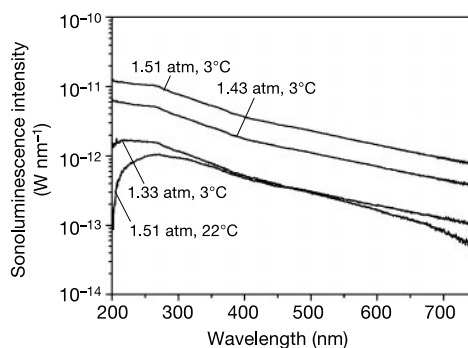


Figure 2 SBSL spectra of water collected at 52 kHz and 3 °C at acoustic intensities of 1.51, 1.43 and 1.33 atm, and at 22 °C and 1.51 atm. Quartz windows were attached to a 15-ml glass cell for better transmittance in the ultraviolet. Spectra were collected at the same acoustic intensities as chemical measurements, that is, at intensities where the bubble can be stable for a long time. Maximum SBSL intensities in the same cells can be ~ 2 to 3 times higher for brief periods of time. SBSL spectra were collected using a Jobin-Ivon Triax-320 monochromator with a spectrum one CCD detector (Instrument SA, 1,024 $\times$ 256 array). Spectra were corrected for light absorbance by water, quartz and the response of the optical system against NIST traceable standard lamps (OLUV-40, Optronics Lab., Inc. and EH-132 Eppley Lab). A long-pass filter was used for the data collection at wavelengths above 400 nm in order to cut off the second-order light. For absolute measurements of SBSL intensity, an OLUV-40 lamp with a neutral density filter (Oriol Corp.) was used.

Table 1 Quantitative sonochemistry in a single cavitation bubble at 52 kHz

Conditions	22 °C	3 °C
R_{max} , μm^*	28.9	30.5
Number of $\text{OH}^\bullet$ radicals per cycle	6.6×10^5	8.2×10^5
Number of photons per cycle	8.1×10^3	7.5×10^4
Number of NO_2^- ions per cycle	3.7×10^6	9.9×10^6
Potential energy at R_{max} (eV)	6.4×10^{10}	7.5×10^{10}
Energy to form $\text{OH}^\bullet$ radicals (eV per cycle) [†]	3.4×10^6	4.3×10^6
Energy to form NO_2^- ions (eV per cycle) [†]	1.6×10^6	4.2×10^6
Energy of photons (200–750 nm) (eV per cycle)	2.7×10^4	2.6×10^5
Energy efficiency of sonoluminescence [‡]	4.3×10^{-7}	3.5×10^{-6}
Energy efficiency of sonochemistry [§]	7.8×10^{-5}	1.1×10^{-4}

*Experimentally observed (see text). Acoustic pressure: 1.5 atm.

†From the enthalpy of formation of $\text{H}_2\text{O} \rightarrow \text{OH}^\bullet + \text{H}^\bullet$ and of $2\text{H}_2\text{O} + 2\text{N}_2 + \text{O}_2 \rightarrow 4\text{HNO}_2$ in the gas phase at 273 K and 1 atm, respectively.

‡Energy of photons/potential energy of bubble.

§Endothermicity of reactions/potential energy of bubble.

Storey and Szeri conducted a detailed theoretical examination of the effect of water vapour on SBSL and sonochemistry¹⁷, and predicted that water vapour must significantly decrease the peak temperature inside the collapsing bubble by reducing compressional heating and through endothermic chemical reactions. They calculated that the temperature inside the bubble is $\sim 7,000$ K, insufficient to cause substantial ionization of molecules, and thus did not incorporate ionization in their model¹⁷. Thus, SBSL can be at least partially due to broadened emission from small molecules, similar to multibubble sonoluminescence². The calculated peak temperature inside the bubble does not change as the compression ratio ($R_{\max}/R_{\min}$) increases: more water is trapped inside the bubble at higher compression ratios, which counteracts the greater energy initially deposited in the larger bubble¹⁷. It has been found in a theoretical paper²⁰ that the excluded volume of the non-ideal gas results in significant suppression of the particle-producing endothermic chemical reactions within the bubble under sonoluminescence conditions. Thus, temperatures of up to 13,000 K can be achieved, which is sufficient for considerable bremsstrahlung emission.

Our data on the yield of nitrite ions during single-bubble cavitation allow us to estimate the diffusion rate of nitrogen inside the bubble and to compare it with theoretical predictions. N_2 , O_2 and Ar will diffuse into a pulsating bubble in water containing dissolved air during the expansion phase. When the bubble compresses, N_2 and O_2 react under the high-temperature conditions inside the bubble and the products of these reactions dissolve in the surrounding water. This selectively leaves Ar inside the bubble, and after many pulsations the bubble will contain primarily Ar (ref. 11). Small amounts of N_2 and O_2 diffuse into the bubble during each expansion. If each molecule of nitrogen gives two molecules of nitrite ions, we can estimate a theoretical yield of nitrite ions from expected diffusion rates. Using a standard diffusion model¹¹, we would expect $\sim 1.2 \times 10^6$ nitrite ions per cycle to be formed in the range 3 °C to 22 °C. This is somewhat low compared to experiment (by factors of 8 and 3 at 3 °C and 22 °C, respectively), probably owing to the neglect of convective flows around the cavitating bubble, which would increase calculated rates of diffusion into the bubble.

Observation of chemical activity from single-bubble cavitation has been claimed recently by LePoint and co-workers^{21,22}, but these authors assert that the chemical yields were uncorrelated with the observation of sonoluminescence. Chemical activity was reported from a single bubble in an aqueous solution of carbon tetrachloride containing a mixture of sodium iodide and starch²¹; however, we observe under similar conditions that such 'bubbles' are actually droplets of CCl_4 whose presence appears to enhance the already facile oxidation of aqueous iodide. The only other report of single-

bubble sonochemistry consists of the formation of solid particles (composition undetermined) during pulsation of a single bubble in an aqueous solution of carbon disulphide²².

The paper by Taleyarkhan *et al.*²³ announcing the observation of D-D nuclear fusion during neutron-induced acoustic cavitation has proved highly controversial²⁴. The authors claim the observation of neutrons and production of tritium after neutron-induced cavitation of perdeuterated acetone (C_3D_6O). Our results raise an important question. Acetone is considerably more volatile than water (30 kPa versus 3.2 kPa at 25 °C; 9.0 kPa versus 0.6 kPa at 0 °C), therefore cavitating bubbles in acetone will contain many more polyatomic molecules. The temperatures reached during cavitation will be substantially limited by the endothermic chemical reactions of the polyatomic molecules inside the collapsing bubble. We therefore expect that the extraordinary conditions necessary to initiate nuclear fusion will be exceedingly difficult to obtain in any liquid with a significant vapour pressure. However, the possibility of such events²⁵ in very low volatility liquids (for example, some polar organic liquids²⁶, molten salts or liquid metals) cannot be ruled out.

The present results show that endothermic sonochemical reactions within a collapsing bubble are a major limitation on the conditions produced during cavitation. □

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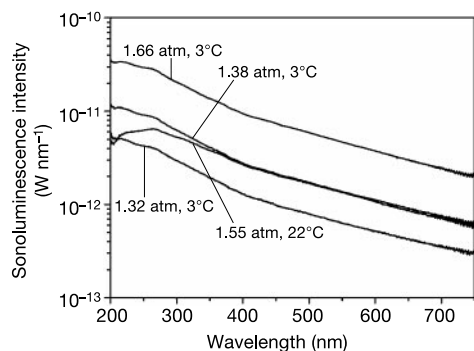


Figure 3 SBSL spectra of water collected at 28 kHz from a 100-ml quartz cell at 3 °C, 1.66 atm; 3 °C, 1.38 atm; 22 °C, 1.5 atm and 3 °C, 1.32 atm.

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Equilibrium lithium transport between nanocrystalline phases in intercalated TiO₂ anatase

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Microcrystalline TiO₂ with an anatase crystal structure is used as an anode material for lithium rechargeable batteries^{1,2}, and also as a material for electrochromic^{3–6} and solar-cell devices^{7,8}. When intercalated with lithium, as required for battery applications, TiO₂ anatase undergoes spontaneous phase separation into lithium-poor (Li_{0.01}TiO₂) and lithium-rich (Li_{0.6}TiO₂) domains on a scale of several tens of nanometres⁹. During discharge, batteries need to maintain a constant electrical potential between their electrodes over a range of lithium concentrations. The two-phase equilibrium system in the electrodes provides such a plateau in potential, as only the relative phase fractions vary on charging (or discharging) of the lithium. Just as the equilibrium between a liquid and a vapour is maintained by a continuous exchange of particles between the two phases, a similar exchange is required to maintain equilibrium in the solid state. But the time and length scales over which this exchange takes place are unclear. Here we report the direct observation by solid-state nuclear magnetic resonance of the continuous lithium-ion exchange between the intermixed crystallographic phases of lithium-intercalated TiO₂. We find that, at room temperature, the continuous flux of lithium ions across the phase boundaries is as high as $1.2 \times 10^{20} \text{ s}^{-1} \text{ m}^{-2}$.

We used ⁷Li magic-angle-spinning (MAS) solid-state NMR, because this microscopic probe gives information on structure and dynamics^{10,11}. The anatase starting material had a particle size of ~10 μm (nitrogen BET specific surface area <0.5 m² g⁻¹). In Fig. 1, the central part of the ⁷Li MAS NMR spectrum of a lithium-intercalated sample, Li_{0.12}TiO₂, is shown. In this sample, two phases coexist⁹: the lithium-poor phase Li_{0.01}TiO₂ with the anatase struc-

ture (space group *I41/amd*), and the lithium-rich phase indicated by lithium titanate (Li_{0.6}TiO₂; space group *Imma*). The Li in anatase gives a sharp line in addition to the broad Li in titanate signal.

The exchange of Li between the two phases is established by performing two-dimensional exchange measurements¹². This technique effectively measures the spectrum of the ⁷Li atoms at $t = 0$ s, then waits a 'mixing time' t_{mix} , and subsequently measures the spectrum of the same ⁷Li atoms again at $t = t_{\text{mix}}$. The results of such measurements are shown in Fig. 2. The signal occurring on the diagonal in the plots represents ⁷Li atoms that have the same spectrum before and after t_{mix} —that is, ⁷Li that remained in the same crystallographic phase. The spectral intensity produced by ⁷Li that is located in anatase at $t = 0$ and in titanate at $t = t_{\text{mix}}$ (or *vice versa*) is at the corresponding off-diagonal positions. The ridges in Fig. 2b are off-diagonal signals of this type; they are strong because of the large amount of Li that has exchanged between the two phases within the diffusion time of $t_{\text{mix}} = 50$ ms.

In Fig. 2a $t_{\text{mix}} = 50 \mu\text{s}$, which means virtually no diffusion time, and thus no exchange. Because about 40% of the intensity in the sharp peak in Fig. 2a is transferred to intensity in the ridges in Fig. 2b, about 40% of the initial amount of ⁷Li in anatase has exchanged with ⁷Li in the titanate phase after $t_{\text{mix}} = 50$ ms, as can be seen when comparing Fig. 2a and b. In Fig. 2c, a measurement is shown at 148 K using a t_{mix} of 50 ms. At this temperature the Li motion between anatase and titanate is frozen. But there still appears to be diffusion within the titanate phase; this will be due to the low activation energy for hopping between sites within this phase⁹. The measurement is shown here to prove that the cross-signal intensities in Fig. 2b cannot arise from spin diffusion due to the presence of dipolar couplings (these are suppressed by MAS).

Faster one-dimensional experiments were performed to quantify the exchange in terms of the magnetization of Li in anatase as a function of t_{mix} and temperature T . The technique is described in Fig. 3 legend. The experimental results in Fig. 3 are given as a function of t_{mix} , and for several temperatures. The data are analysed using the solution of Fick's law of diffusion, $\partial m(\mathbf{r}, t) / \partial t = \nabla \cdot \{D(\mathbf{r})m(\mathbf{r}, t)\}$, where $m(\mathbf{r}, t)$ is the magnetization of Li at position $\mathbf{r}$ and t , and D is the Li diffusivity. Best results were obtained with

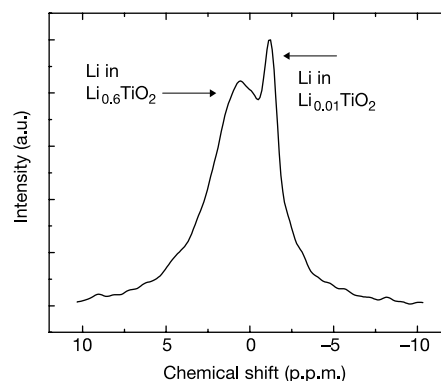


Figure 1 Central part of the ⁷Li magic-angle-spinning NMR spectrum of Li_{0.12}TiO₂ at 100 °C, showing the resonances of Li in the two coexisting phases. A spinning speed of $20,000 \pm 3$ Hz and a radio-frequency field strength corresponding to a ⁷Li spin nutation frequency of 192 kHz was used on a Chemagnetics 600 spectrometer. Two phases are coexisting in thermal equilibrium: a Li-poor anatase phase with a composition of Li_{0.01}TiO₂ (narrow peak) and a Li-rich titanate phase with composition Li_{0.6}TiO₂ (this peak is homogeneously broadened, with the transverse relaxation time T_2 giving its linewidth, indicating that the ⁷Li in this phase are all experiencing similar environments).

isotropic diffusion in three dimensions, which agrees with the data of ref. 13, and the single-crystal data of ref. 14. Using the mathematics of the models for spin diffusion^{15,16}, and assuming (for simplicity) the overall diffusivity to be isotropic and spatially independent, the rate of demagnetization of a cube of phase A (anatase), initial magnetization $m_{A,0}$, and dimension d_A , owing to diffusion of magnetized Li towards the surrounding unmagnetized

phase B (titanate), can be derived as:

$$m_A(t_{\text{mix}}) = \frac{m_{A,0}}{(c_A + c_B)^3} \left\{ 2d_A c_A + c_B \sqrt{4Dt_{\text{mix}}} \left[\text{ierfc} \left(\frac{d_A}{\sqrt{4Dt_{\text{mix}}}} \right) + \text{ierfc} \left(\frac{-d_A}{\sqrt{4Dt_{\text{mix}}}} \right) - \frac{2}{\sqrt{\pi}} \right] \right\}^3$$

Here $\text{ierfc}(x) = (1/\sqrt{\pi}) \exp(-x^2) - x(1 - \text{erf}(x))$, and $c_A^3 = 0.01$ and $c_B^3 = 0.60$ represent the Li concentrations in anatase and titanate, respectively, which were determined previously⁹. The dimension of the anatase domains was determined⁹ from the width of X-ray diffraction peaks to be about 60 ± 15 nm. The only remaining fitting parameter in the model is the diffusion rate, D , for the diffusion of Li in the anatase and titanate system.

The fits are shown in the top panel of Fig. 3. The bottom part of Fig. 3 gives an Arrhenius plot of the temperature dependence of D . An activation energy of 0.45 ± 0.06 eV can be deduced for the overall cross-boundary diffusion, which is close to the value found from macroscopic Li diffusion data¹³. Recent first-principles calculations yielded a similar value for the activation energy for the growth of the Li-rich phase on Li intercalation¹⁷.

The current density of Li flowing back and forth between the two phases can be determined from the timescale at which Li crosses the boundary, and from the surface areas of the ~ 60 -nm-diameter spheres of anatase. At room temperature a value of $1.2 \times 10^{20} \text{ s}^{-1} \text{ m}^{-2}$ is calculated. At 373 K the system is clearly highly dynamic on timescales much greater than 50 ms, and we suggest that the boundaries of the two phases will be non-stationary if not every Li diffusing to the other phase is replaced at exactly the same position. On such timescales, this would lead to diffusive

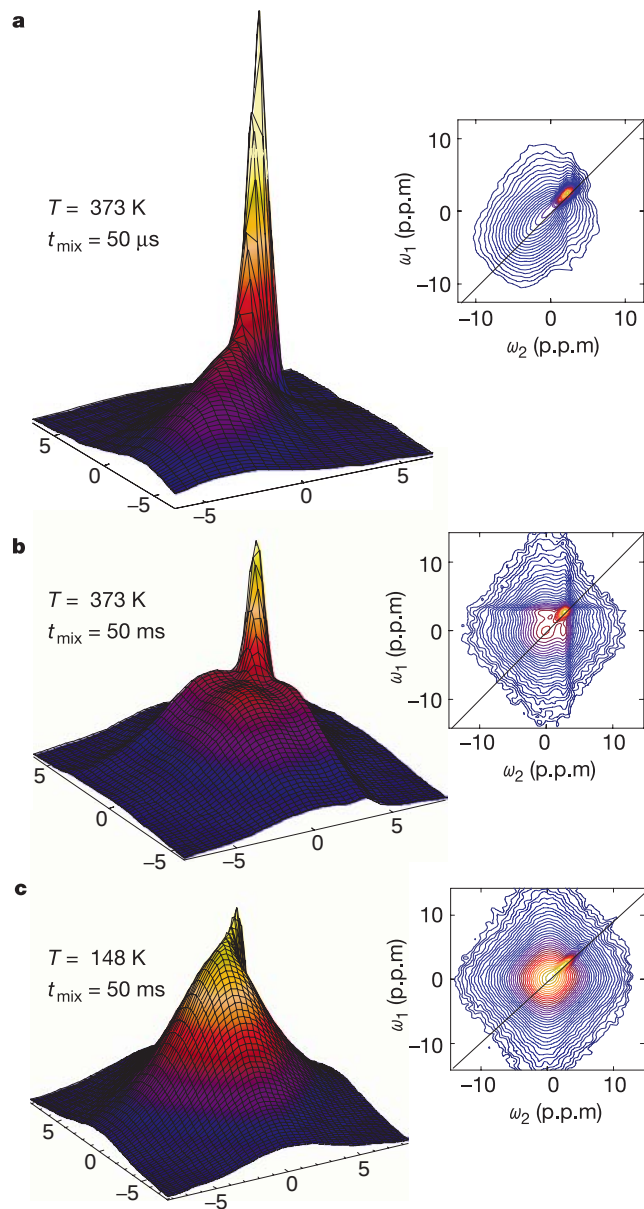


Figure 2 Exchange of Li between the two coexisting phases in $\text{Li}_{0.12}\text{TiO}_2$ measured with ^7Li two-dimensional exchange NMR. The contour plots (insets) are projections of the three-dimensional representations; ω_1 and ω_2 represent the chemical shift before and after t_{mix} respectively. **a**, At $T = 373$ K and $t_{\text{mix}} = 50 \mu\text{s}$ (see text) there are no cross-signal intensities visible, indicating that the diffusion time of $50 \mu\text{s}$ is still too short for cross-boundary lithium diffusion. **b**, At $T = 373$ K and $t_{\text{mix}} = 50$ ms, there are large ridges visible covering the widths of the Li in titanate signal at the position of the Li in anatase signal. These cross-signal intensities are a direct measure of cross-boundary Li diffusion. **c**, At $T = 148$ K and $t_{\text{mix}} = 50$ ms, the cross-boundary lithium diffusion is absent owing to freezing of Li motion in the anatase phase. The rectangular off-diagonal shape of the Li in titanate peak shows that there is still diffusion within the titanate phase.

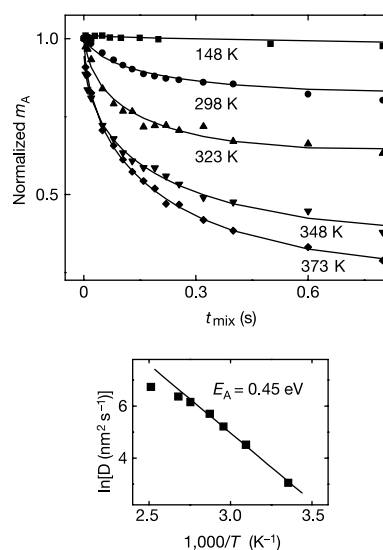


Figure 3 Quantifying the diffusion process. Top, time dependence of ^7Li magnetization in the anatase phase, indicating Li diffusion towards the titanate phase. The one-dimensional NMR pulse sequence $\pi/2, \tau, \pi, \tau, -\pi/2, t_{\text{mix}}, +\pi/2$, acquisition, was used, with the appropriate phase cycles for cancellation of direct magnetization that may occur after T_1 relaxation. (T_1 is the longitudinal relaxation time.) Using appropriate values of τ , the intensity of the narrow anatase line was conserved while that of the broad titanate line vanishes (T_2 filtering): that is, before t_{mix} starts, magnetization is only present in the anatase phase. Diffusion of lithium into the titanate phase during t_{mix} causes the normalized intensity $m_A(t_{\text{mix}})$ of the narrow anatase line to decrease strongly as a function of t_{mix} , while the broad titanate line becomes populated again. The lines represent fits using a Li diffusion model described in the text. Bottom, the temperature dependence of the diffusion parameter D . Using an Arrhenius behaviour, an activation energy E_A of 0.45 ± 0.06 eV is determined for the cross-boundary diffusion process.

motion of crystalline domains in an otherwise rigid solid-state system. □

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Synthetic hosts by monomolecular imprinting inside dendrimers

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Synthetic host systems capable of selectively binding guest molecules are of interest for applications ranging from separations and chemical or biological sensing to the development of biomedical materials. Such host systems can be efficiently prepared by 'imprinting' polymers or inorganic materials with template molecules, which, upon removal, leave behind spatially arranged functional groups that act as recognition sites^{1–4}. However, molecularly imprinted polymers have limitations, including incomplete template removal, broad guest affinities

and selectivities, and slow mass transfer^{5–8}. An alternative strategy for moulding desired recognition sites uses combinatorial libraries of assemblies that are made of a relatively small number of molecules, interconverting in dynamic equilibrium; upon addition of a target molecule, the library equilibrium shifts towards the best hosts^{9–11}. Here we describe the dynamic imprinting of dendritic macromolecules with porphyrin templates to yield synthetic host molecules containing one binding site each. The process is based on our general strategy to prepare cored dendrimers^{12,13}, and involves covalent attachment of dendrons to a porphyrin core, cross-linking of the end-groups of the dendrons, and removal of the porphyrin template by hydrolysis. In contrast to more traditional polymer imprinting, our approach ensures nearly homogeneous binding sites and quantitative template removal. Moreover, the hosts are soluble in common organic solvents and amenable to the incorporation of other functional groups, which should facilitate further development of this system for novel applications.

When developing our 'monomolecular imprinting' methodology, we selected dendrimers as macromolecular hosts because their molecular homogeneity and excellent solubility facilitate characterization¹⁴. The choice of a porphyrin as templating agent was motivated by the scarce availability of synthetic hosts for large molecule guests¹⁵ and the fact that porphyrins are excellent probes of binding, owing to the sensitivity of their intense colour to the local environment¹⁶. Porphyrins have been integrated into molecularly imprinted polymers¹⁷ and synthetic hosts¹⁸, but have to our knowledge not been used as templates. Moreover, the chemistry of porphyrin-core dendrimers is well established, given their role as well-studied synthetic models of haem proteins and related proteins^{19–22}.

The basic strategy for synthesizing dendrimer hosts follows our earlier work on cored dendrimers^{12,13}. The preparation of porphyrin-core dendrimer **1** is summarized in Fig. 1. The key structural features of this pre-cross-linked dendrimer are the tetrakis-meso(3,5-dihydroxyphenyl)-porphyrin (**3**)¹⁹ unit at the core, and hydrolysable ester-bond links to eight third-generation Fréchet-type dendrons²³ (**2**)¹², which give a total of 64 homoallyl end-groups (see Supplementary Information). In addition to **1**, a small amount (~5%) of dendrimers containing six or seven dendrons was formed (see below).

Porphyrin-core dendrimer **1** was cross-linked¹² using 4 mol% (per alkene) of Grubbs' catalyst **4**²⁴ at 10^{–6} M in benzene to produce cross-linked dendrimer **5** in 88% yield. Size exclusion chromatography (SEC) established that cross-linking occurs largely (≥95%) intramolecularly. The ¹H NMR spectrum of **5** confirmed the formation of internal, disubstituted alkene groups and gave the average number of cross-links as approximately 29. The matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrum of **5** provided conclusive evidence for extensive intramolecular cross-linking: it contained peaks corresponding to individual isomers from 28 to 32 out of 32 possible cross-links (mass/charge ratios (*m/z*) 10,369, 10,341, 10,313, 10,285, 10,257), with the tallest peak at *m/z* 10,285 (31 cross-links, see Supplementary Information). The ability of catalyst **4** to produce from **1** a compact structure with a median of 30 out of 32 possible cross-links may be due to the reversibility of the ring closing metathesis (RCM) reaction²⁴. The precise details of the cross-linking process remain to be determined. But analogy with the quantitative RCM of polyolefins²⁵ suggests that it may be a dynamic process, thus ensuring that millions of kinetically favoured cross-link isomers may equilibrate into a small number of thermodynamically stable ones.

Treatment of the cross-linked dendrimer **5** with 2.5 M aqueous KOH solution in tetrahydrofuran (THF) quantitatively removed the porphyrin template (that is, **3**), forming **6** in a 43% yield. The ¹H NMR spectrum of **6** showed no signals originating from the porphyrin core, and the UV–vis. spectrum of **6** showed no detectable absorbance at 420 nm, the Soret band of the porphyrin. Elemental analysis of the hydrolysis product was consistent with a

dodecahydrate of **6** containing 30 cross-links. Elemental analysis for both nitrogen and ruthenium showed none present ($0.0 \pm 0.3\%$) and only a trace of potassium. Finally, comparison of the MALDI-TOF mass spectra of **5** and **6** showed the peaks in the latter shifted to lower m/z values by 597 (see Supplementary Information), corresponding to the loss of the porphyrin core ($C_{44}H_{14}N_4$; m/z 598.6).

The hydrolysis reaction that removes the template molecule $H_2T(3,5-OHPh)P$ (**3**) results in the addition of eight $-OH$ groups inside the dendrimer host, so **3** does not generate an ideal imprint for binding of itself (Fig. 2). In the limit of a well-formed imprint, the hydrolysis reaction creates a binding site that is too small to accommodate **3**, but might be suitable for the isomeric hydroxy compound, $H_2T(2,6-OHPh)P$ (**7**) or tetrapyrimidyl porphyrin ($H_2T(3,5\text{-pyrimidyl})P$) (**8**). An extensive series of porphyrins, **7–16** (Fig. 3), was therefore prepared^{26,27}, and used to study the binding of imprinted dendrimer **6**.

To conduct the binding experiments, the solvent had to be sufficiently apolar to allow formation of hydrogen bonds, but polar enough to dissolve both **6** and the porphyrin. The complexation studies with octahydroxyporphyrins **3** and **7** were performed in 5% (v/v) ethyl acetate–toluene, whereas **8–16** were sufficiently soluble to study in toluene. Spectrophotometric titrations were performed by adding aliquots of a concentrated solution of imprinted dendrimer to a dilute solution of porphyrin and recording the Soret region after 10 minutes of equilibration.

Complexation of porphyrin **7** by imprinted dendrimer **6** was signalled by a significant bathochromic (red) shift in the λ_{max} of the Soret band of **7** (3 μM solution in 5% ethyl acetate–toluene) upon addition of **6** (see Supplementary Information). Consistent with

hydrogen-bond complexation, the red shift could be fully reversed by increasing the ethyl acetate content from 5% (v/v) to >15% (v/v) in toluene. Furthermore, no red shift was observed when **6** was added to a solution of **7** in 50% (v/v) ethyl acetate–toluene. In 5% ethyl acetate–toluene no change in the Soret band of H_2TPP (**9**) or $H_2T(3,5-OHPh)P$ (**3**) occurred upon addition of **6**. The latter result argues for a relatively rigid imprint; that is, **6** is not a flexible octa-acid that can complex any guest with sufficient hydrogen bond donors or acceptors. The magnitude of the shift in the Soret band of **7** was directly dependent on the amount of **6** added and the time elapsed. Extensive studies to be published separately indicate a relatively homogeneous imprint that produces within two hours a complex with a 5 nm red shift and a diminished apparent extinction coefficient, which, in turn, undergoes a slow conformation change over 17 days producing a tighter complex (10-fold increase in apparent association constant K_{app}) with an additional 6 nm red shift and an increased apparent extinction coefficient. The results described below are for the fast binding process only.

Complexation of $H_2T(2,6-OHPh)P$ (**7**) by imprinted dendrimer **6** was also observed by SEC. Porphyrin **7** does not elute from the SEC matrix by itself, but in the presence of the imprinted dendrimer **6** it elutes with a measured retention time identical to that of the dendrimer. Thus, complexation within the dendrimer protects the polar porphyrin from adsorption during the course of its elution and decomplexation is slow on the timescale of the SEC experiment (minutes).

To determine the shape selectivity and number of binding contacts to the ligand, quantitative binding studies were carried out with porphyrins **7–16** (Fig. 3) using standard methods²⁸. The

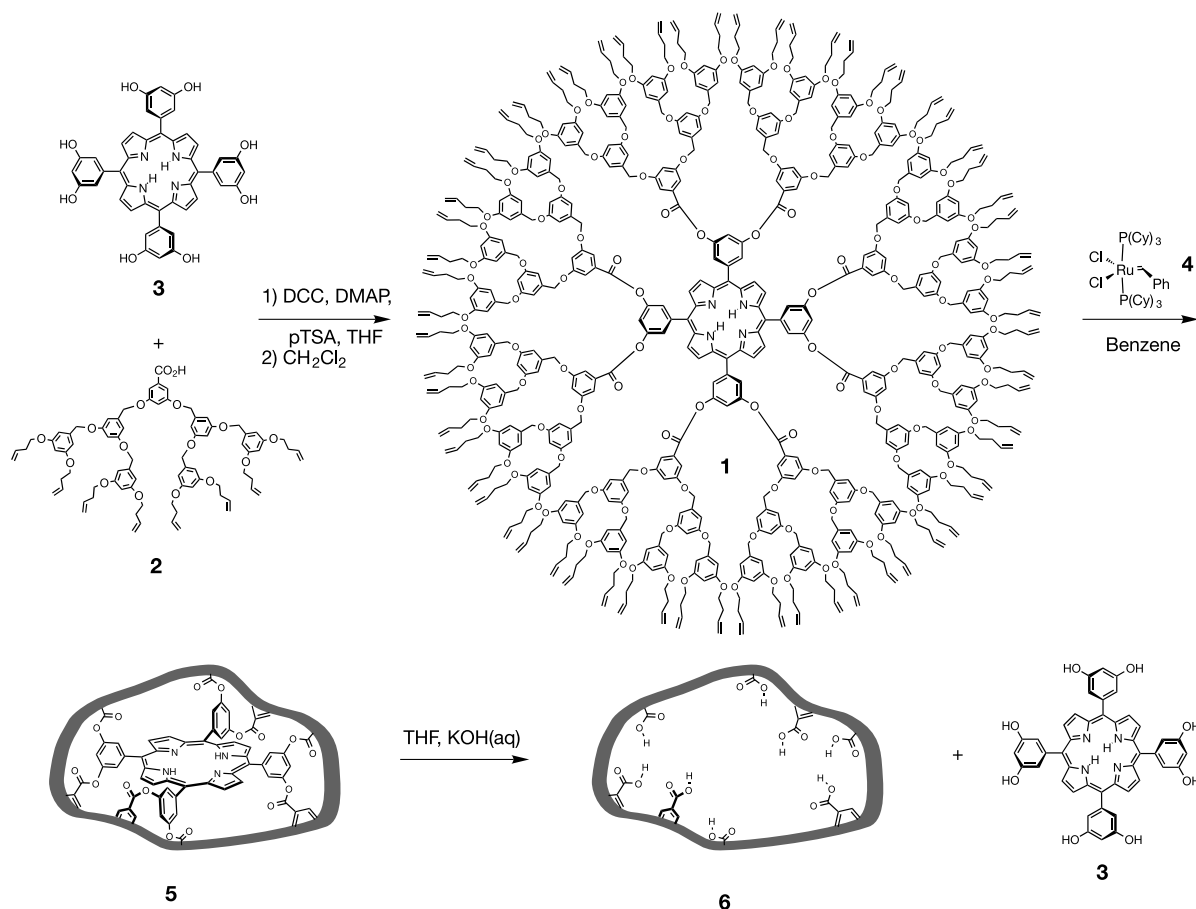


Figure 1 Scheme illustrating the preparation of imprinted dendrimer **6**. First, dendrons **2** are attached to the core porphyrin **3** to produce the dendrimer **1**, then **1** is cross-linked using Grubbs' catalyst **4** to give the dendrimer **5**, followed by the hydrolysis of **5** to remove

the porphyrin core **3** and produce **6**. DCC, dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; pTSA, *p*-toluene sulphonic acid; THF, tetrahydrofuran.

association constants (Fig. 3) are denoted as K_{app} because of the likely heterogeneity in **6** and time dependence of the binding process. $H_2T(3,5\text{-pyrimidyl})P$ (**8**) is bound by **6** with a K_{app} ($5 \times 10^4 M^{-1}$) of the same magnitude as the **6-7** association. The pyridyl porphyrins $H_2T(2\text{-pyridyl})P$ (**10**), $H_2T(3\text{-pyridyl})P$ (**11**) and $H_2T(4\text{-pyridyl})P$ (**12**) bind equally well, despite their capacity to make only half as many hydrogen bonds as **8**. Although this may suggest that the full eight carboxylic acids are not simultaneously engaged in binding $H_2T(3,5\text{-pyrimidyl})P$ (**8**), the difference must partly originate in the 10^4 -fold higher basicity of pyridine relative to pyrimidine. Along the same lines, given that typical pyridine-carboxylic acid association constants in apolar organic solvents are $\sim 10^2 M^{-1}$, it might be expected that complexes containing at least four such interactions would exhibit absolute K_{app} values higher than 10^4 to $10^5 M^{-1}$. Factors that may lower the K_{app} values for **6** include the need to break any internal carboxylic acid dimers or otherwise conformationally reorganize the binding site, and remove water molecules bound to the carboxylic acid groups. Although infrared studies of **6** were inconclusive with respect to possible dimerization of its carboxylic groups, elemental analysis of **6** indicates the presence of water presumably solvating the polar core. The K_{app} for the **6-10** (2-pyridyl isomer) is approximately 2-fold lower than that for the 3- and 4-pyridyl isomers **11** and **12**, indicating a degree of shape selectivity in the imprint. No binding was detected for tetraphenyl, mono, bis, and trispyridyl porphyrins **9**, **13**, **14**, **15** or **16**. Thus, a minimum of four binding contacts is necessary for complexation under the conditions used in this study.

To further support the importance of reversible complexation by hydrogen bonding and the direct involvement of the carboxylic acid groups, the octa-ethyl ester dendrimer **6**-(CO₂Et)₈ was prepared by ethanolysis of **5** (K₂CO₃, toluene, ethanol, reflux). The structure of **6**-(CO₂Et)₈ was verified by SEC, ¹H NMR spectroscopy, MALDI-TOF mass spectrometry, and UV-vis. spectroscopy. The addition of approximately 60 equivalents of **6**-(CO₂Et)₈ to $H_2T(3\text{-pyridyl})P$ (**11**) caused no red shift over the course of 4 days. Likewise addition of simple carboxylic acids did not cause a red shift of $H_2T(3\text{-pyridyl})P$ (**11**). Neither the addition of 640 equivalents of dendron carboxylic acid **2** to a $\sim 3 \mu M$ solution of $H_2T(2,6\text{-OHPh})P$ (**7**) in 5% ethyl acetate-toluene nor the addition of 2,000 equivalents of 4-*t*-butyl benzoic acid to a $\sim 3 \mu M$ solution of $H_2T(3\text{-pyridyl})P$ (**11**) in toluene led to any red shift. Finally, a smaller dendrimer with three acid groups, which was imprinted with 1,3,5-tri(hydroxymethyl)benzene¹², did not alter the position of the Soret band of a $\sim 3 \mu M$ solution of **11** in toluene even upon addition of 49 equivalents.

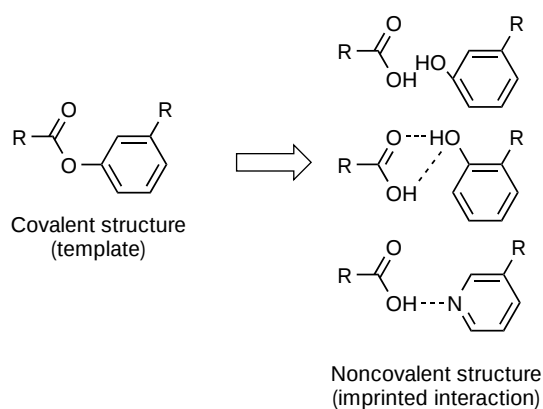


Figure 2 Structural drawing illustrating structural changes that occur upon hydrolysis of the cross-linked dendrimer **5**. The covalent structure contains eight phenyl benzoates formed by esterification of the phenolic porphyrin **3** with the carboxylic acid dendrons **2**. Note that two oxygen atoms would be left in the same space if both components of the covalent structure were held fixed. The isomeric phenol is better accommodated and phenyl benzoate appears to imprint well for a 3-pyridyl or 3,5 pyrimidyl porphyrin.

As indicated above, a known source of heterogeneity in the imprinted dendrimer arises from incomplete reaction of **2** with **3**. This imperfect material was shown not to play a role in binding by carefully removing it with repeated preparative SEC. A different type of heterogeneity may originate in the RCM reaction of dendrimer **1**, which can produce **5** containing a very large number of cross-link isomers. The broad peaks observed in the ¹H NMR of **5** and **6** might be attributed to the presence of a mixture of such isomers, although the heterogeneity does not manifest itself in the binding (see above).

To gain insight into the three-dimensional structure of **6** and how it changes with the extent and position of the cross-links, molecular modelling was performed using molecular mechanics and dynamics. A series of cross-linked dendrimers **5** were built and minimized. The porphyrin core was removed, providing a globular porous structure with eight carboxyl groups in its interior. The resulting models of **6** were subjected to 60 ps of molecular dynamics alone and with $H_2T(3,5\text{-pyrimidyl})P$ (**8**) within the binding site. In both cases the overall globular shape of **6** did not significantly change compared to that of **5**. The van der Waals surface of a modelled imprinted dendrimer with nine intra- and 20 inter-dendron cross-links (Fig. 4a) reveals a slightly porous structure with no obvious large channels to the binding site. Thus, complexation and decomplexation must involve a dynamic breathing process (in other models with a higher fraction of intra-dendron cross-

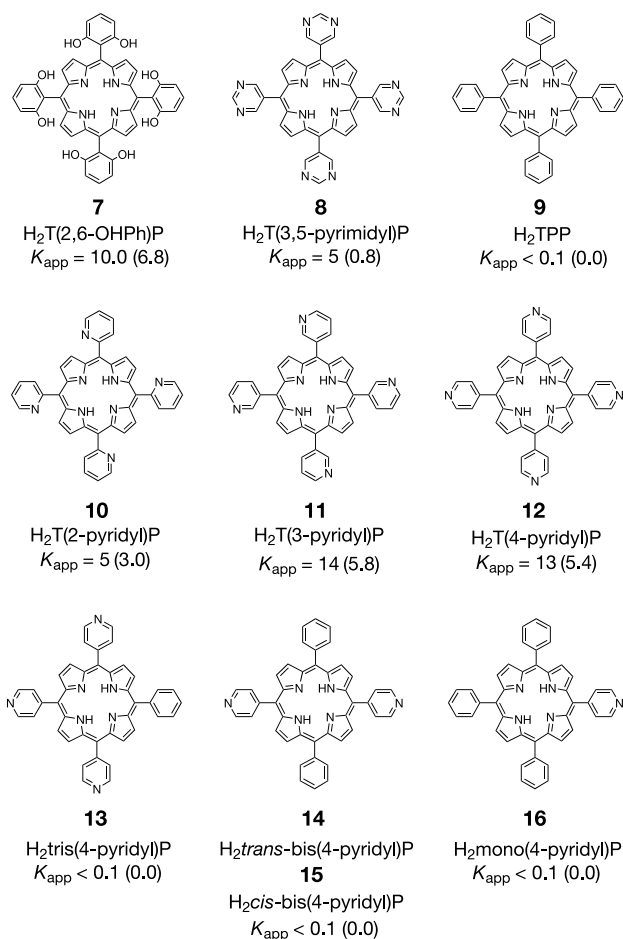


Figure 3 Porphyrins used to study binding properties of imprinted dendrimer **6**. Each structure is accompanied by the corresponding apparent association constant K_{app} ($\times 10^4 M^{-1}$), and the red shift value of the Soret band ($\Delta\lambda_{max}$, nm) for the complex formed between imprinted dendrimer **6** and the porphyrin. A value of $\Delta\lambda_{max} = 0.0$ indicates no observed change in the absorption spectrum. Upper limits on solubility prevented a more accurate upper limit of K_{app} from being obtained.

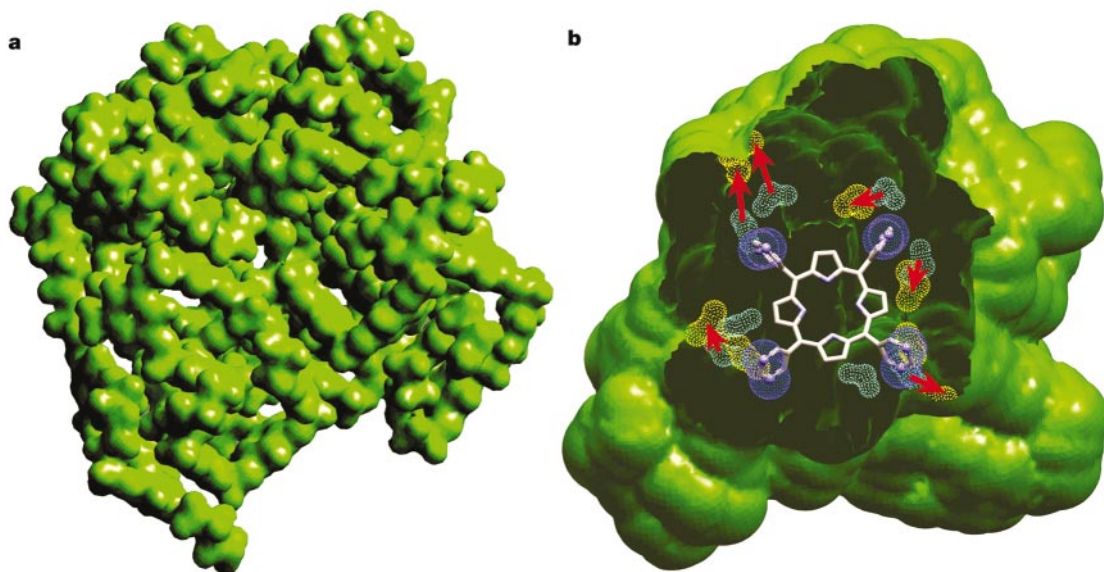


Figure 4 Calculated structure of imprinted dendrimer **6** built by iterative attachment of dendrons to the core porphyrin, minimization and cross-linking of neighbouring double bonds. **a**, van der Waals surface of a representative dendrimer structure produced by a 60 ps annealed dynamics simulation. **b**, Calculated binding pocket inside **6**; yellow clouds

links, the binding pocket appears more readily accessible). The carboxyl groups in **6** moved from their original positions in the ester links of **5**, but the binding pocket was mostly intact (Fig. 4b). The displacement of carboxyl groups was less significant when **8** was present in the interior of the cored dendrimer as seen in the overlaid structures in Fig. 4b.

As a demanding test of the monomolecular imprinting process, we chose a particularly large molecule guest, a polyfunctionalized porphyrin, as the template within an octa-dendron array. Extensive cross-linking using the RCM reaction followed by template removal produced an imprinted dendrimer that selectively and tightly ($K_{app} = (0.5-1.4) \times 10^5 M^{-1}$) bound porphyrins presenting an appropriate size and four or more hydrogen-bonding sites (for example, **7**, **8**, **10-12**). The binding is size selective on the basis of results with the octahydroxyl porphyrins **3** and **7**, and modestly shape-selective on the basis of the results with the isomeric pyridyl porphyrins (**10-12**).

The limitations of this approach are the need for multi-step synthesis of the dendrimers, and the high dilution conditions needed for the RCM reaction. (We note that it was recently shown that this high dilution is not necessary for a dendrimer where the alkene groups reside in the branching units¹³.) But even in its current implementation, the monomolecular imprinting approach has several characteristics not yet obtainable through traditional polymer imprinting. These include high-efficiency imprinting with nearly all templates producing functional binding sites, quantitative removal of the template, solubility of the imprinted material in common organic solvents, and separation of imperfectly assembled binding sites. By using resins covalently linked to the target ligand, it should be possible to fractionate heterogeneous material after imprinting. In this manner, selection of imprinted sites on the basis of affinity or binding kinetics should be possible. Finally, the single-step synthesis of hyperbranched or analogous macromolecules, combined with the ability to integrate reporter groups, makes the monomolecular imprinting approach a promising one for further development. □

Methods

The titration data were analysed by plotting $[ID]$ against $[ID]/\Delta A$, where $[ID]$ is the imprinted dendrimer concentration and ΔA is the change in absorbance upon addition of

represent positions of carboxyl groups when **6** is minimized without the host porphyrin **8**, cyan clouds represent positions of carboxyl groups when **6** is minimized in the presence of the host porphyrin **8**, red arrows show the movement of carboxylic groups.

6. This method allows association constants to be determined by finding the line intercept that gives $1/K_{app}$ and does not require knowledge of the concentration of the free or complexed porphyrin. The titrations were all completed within 1 h and all gave linear plots. As with other Scatchard-type plots, linearity indicates the formation of 1:1 complexes.

Molecular mechanics and dynamics were performed using Cerius², version 4.5 (Accelrys). For energy minimizations and simulated annealing (60 ps, 300–500 K), the Dreiding force field was used.

Preparation of **1**

Compound **1** (5,10,15,20-tetrakis[3,5-bis[3,5-bis(3,5-bis(3-buten-1-oxy)benzyloxy)benzyloxy]benzyloxy]phenyl)porphyrin ([G-3]₈-T(3,5-OHPh)P)) was synthesized as follows. [G-3]-CO₂H (**2**) (283 mg, 215 μmol), 13.2 mg (17.8 μmol) of **3** (5,10,15,20-tetrakis(3',5'-dihydroxyphenyl)porphyrin, referred to as H₂T(3,5-OHPh)P), 519 mg (2.5 mmol) of dicyclohexylcarbodiimide (DCC), 78.6 mg (643 μmol) of 4-(dimethylamino)pyridine (DMAP) and 118 mg (621 μmol) of *p*-toluene sulphonic acid (pTSA) were dissolved in 15 ml of THF. The reaction mixture was stirred overnight at room temperature and filtered to remove the dicyclohexylurea (DCU) formed. The filtrate was concentrated under reduced pressure and dissolved in 15 ml of CH₂Cl₂. To this solution, 510 mg (2.5 mmol) of DCC, 75.8 mg (620 μmol) of DMAP and 117 mg (615 μmol) of pTSA was added, and the mixture was stirred at room temperature. The reaction was complete after three days (by thin-layer chromatography and SEC). The reaction mixture was filtered to remove the DCU, and the resulting filtrate was concentrated under reduced pressure. The remaining residue was loaded onto a silica gel column (4 × 15 cm) and eluted with 30% EtOAc/PE. The product was further purified by loading it onto a SEC column and eluting it with toluene. The product was dried overnight under vacuum to afford 107 mg (54%) of **1** as a deep red oil: ¹H NMR (CD₂Cl₂) δ 9.22 (s, 8H), 8.15 (d, 8H), 7.75 (t, 4H), 7.54 (d, 16H), 6.81 (t, 8H), 6.64 (d, 32H), 6.48 (m, 80H), 6.31 (t, 32H), 5.81 (ddt, 64H), 5.07 (ddt, 64H), 5.01 (ddt, 64H), 4.98 (s, 32H), 4.87 (s, 64H), 3.88 (t, 128H), 2.42 (tddd, 128H), -2.86 (s, 2H); ¹³C NMR (CD₂Cl₂) 165.1, 160.7, 160.5, 160.4, 150.3, 144.2, 139.6, 139.3, 135.0, 131.6, 126.3, 118.9, 117.0, 109.3, 108.1, 106.7, 106.2, 101.9, 101.0, 70.5, 70.3, 67.6, 33.9; MS (MALDI-TOF) *m/z* 11,174.7 (M + Na⁺); SEC (toluene) calculated *M_w* = 8,155; UV-vis. (CH₂Cl₂) λ_{max} = 275.0, 420.5 (Soret). Analysis: calculated for C₆₉₂H₇₃₄N₄O₁₂₈: C, 74.51; H, 6.63; N, 0.50. Found: C, 74.56; H, 6.41; N, 0.48.

Cross-linked [G-3]₈-T(3,5-OHPh)P (**5**)

To a solution of 497 mg (44.6 μmol) of **1** in 4.4 litres of benzene was added 99.6 mg (120 μmol) of Grubbs' catalyst (**4**). The reaction mixture was stirred at room temperature for 22 h. The benzene was removed under reduced pressure. The crude product was loaded onto a silica gel plug (4 × 6 cm) and eluted with 400 ml of 50% petroleum ether/50% CH₂Cl₂ and 400 ml of 5% EtOAc/CH₂Cl₂. The EtOAc/CH₂Cl₂ solution was concentrated under reduced pressure. The product was dried overnight under vacuum to afford 409 mg (89%) of **5** as a dark red powder: ¹H NMR (*d*₈-toluene) δ 9.17 (bs, 8H), 8.15 (bs, 8H), 7.70 (bs, 20H), 6.59 (bs, 152H), 5.78 (bs, ~4H), 5.51 (bs, ~60H), 5.04 (bs, ~8H), 4.82 (bs, 96H), 3.70 (bs, 128H), 2.33 (bs, 128H); MS (MALDI-TOF) *m/z* 10,258.2 (M + H⁺ - 32C₂H₄), 10,286.3 (M + H⁺ - 31C₂H₄), 10,311.8 (M + H⁺ - 30C₂H₄); SEC (toluene) calculated *M_w* = 5509; UV-vis. (CH₂Cl₂) λ_{max} = 276.0, 421.0 (Soret).

Imprinted [G-3]₈-T(3,5-OHPh)P (6)

To a solution of 102 mg (9.88 μ mol) of **5** dissolved in 15 ml of THF, was added 10 ml of 2.5 M aqueous KOH. The reaction mixture was stirred vigorously at reflux until the reaction was complete by TLC. The reaction was stopped by removing the THF under reduced pressure. The resulting aqueous layer was extracted with CHCl₃ (3 $\times$ 25 ml). The combined organic layers were washed with 1 M aqueous HCl and water, and concentrated under reduced pressure. The product was dried under vacuum to afford 41.5 mg (43%) of **6** as a beige powder: ¹H NMR δ 7.23 (bs, 16H), 6.51 (bs, 152H), 5.86 (bs, $\sim$ 4H), 5.60 (bs, $\sim$ 60H), 5.12 (bs, $\sim$ 8H), 4.86 (bs, 96H), 3.92 (bs, 128H), 2.45 (bs, 128H); MS (MALDI-TOF) *m/z* 9,710.8 (M + Na⁺ – 31C₂H₄ – C₄₄H₁₄N₄), 9,756.0 (M + K⁺ – 30C₂H₄ – C₄₄H₁₄N₄), 9,779.8 (M + K⁺ – 29C₂H₄ – C₄₄H₁₄N₄), 9,810.7 (M + K⁺ – 28C₂H₄ – C₄₄H₁₄N₄). Analysis: calculated for C₅₈₈H₆₀₀O₁₂₈: C, 72.70; H, 6.22; N, 0.00; Ru, 0.00. Found: C, 70.83; H, 6.19; N, 0.00; Ru, 0.00.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Tungsten isotope evidence from ~3.8-Gyr metamorphosed sediments for early meteorite bombardment of the Earth

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The 'Late Heavy Bombardment' was a phase in the impact history of the Moon that occurred 3.8–4.0 Gyr ago, when the lunar basins with known dates were formed^{1,2}. But no record of this event has yet been reported from the few surviving rocks of this age on the Earth. Here we report tungsten isotope anomalies, based on the ¹⁸²Hf–¹⁸²W system (half-life of 9 Myr), in metamorphosed sedimentary rocks from the 3.7–3.8-Gyr-old Isua greenstone belt of West Greenland and closely related rocks from northern Labrador, Canada. As it is difficult to conceive of a mechanism by which tungsten isotope heterogeneities could have been preserved in the Earth's dynamic crust–mantle environment from a time when short-lived ¹⁸²Hf was still present, we conclude that the metamorphosed sediments contain a component derived from meteorites.

It is widely conjectured that the Earth suffered contemporaneous Late Heavy Bombardment (LHB) with the Moon, scaled up proportionally to its much greater gravitational cross-section. Earth's estimated³ mass accretion rate during the LHB was of the order of (1–2) $\times 10^{15}$ g yr^{–1}, or (2–4) $\times 10^{-4}$ g cm^{–2}. Over a 100-Myr period of LHB, this accretion rate would have yielded (1–2) $\times 10^{23}$ g of material. If added as a continuous veneer over the entire planet, this would correspond to 200 t m^{–2}. The distinct deuterium/hydrogen ratio of the terrestrial hydrosphere⁴ argues against significant cometary accretion, whereas compositions of projectiles in lunar impact melts⁵ indicate infall from asteroids of enstatite chondrite or iron meteorite parentage. Thus, unless the Earth's early crust was continually recycled into the mantle, it should be possible to detect chemical 'fingerprints' of the LHB in the oldest terrestrial sediments.

In a recent study⁶, the extinct radioactive decay scheme ⁵³Mn–⁵³Cr (half-life, 3.7 Myr) was used to detect meteoritic infall in Cretaceous/Tertiary (K/T) boundary sediments. As Cr isotopes in all classes of meteorites and terrestrial samples are distinct, discovery of a non-terrestrial Cr isotope composition⁶ confirmed extraterrestrial origin. A similar opportunity for detecting meteoritic infall is afforded by the ¹⁸²Hf–¹⁸²W system (half-life, 9 Myr). By analogy with the homogeneous terrestrial Cr isotope composition⁶, any variations in W isotope composition that may have existed in the first ~60 Myr of Earth's history would have been homogenized by convection in the early mantle. Therefore the terrestrial W isotope composition is constant^{7,8}. Indeed, all our terrestrial samples other than the early Archaean metamorphosed sediments (metasediments) yield a constant ¹⁸²W/¹⁸³W ratio (Fig. 1 and Table 1) of 0.10 $\pm$ 0.22 ϵ_W units (where ϵ_W is the deviation, in 0.1‰, from the terrestrial ¹⁸²W/¹⁸³W ratio), identical to our standard (ACQUIRE-W), which was reproduced at 0.00 $\pm$ 0.10 ϵ_W .

In contrast, a well-resolved W isotope variability exists among different classes of meteorites. Iron meteorites⁹ have much lower ¹⁸²W/¹⁸³W ratios (Fig. 1) of, on average, –3.7 ϵ_W , reflecting removal of metal-loving W from silicate into the core of planete-

simals when most ^{182}W was still present. Enstatite chondrites¹⁰ yield ϵ_{W} of -2.2 , and differentiated basaltic meteorites, such as eucrites, contain radiogenic W (ref. 11) with ϵ_{W} up to $+39$. Although less reproducible, ordinary chondrites^{12,13} also show resolvably less radiogenic W from -2.0 to -0.64 ϵ_{W} compared to the accessible Earth. There is disagreement about the W isotope composition of carbonaceous chondrites, but the most recent determinations^{7,13} in the carbonaceous chondrite Allende yielded -2.2 and -2.0 ϵ_{W} . Thus it appears that all classes of meteorites have W isotope compositions different from terrestrial samples, and deviations from the terrestrial W isotope composition necessarily imply extraterrestrial W.

The six 3.7–3.8-Gyr amphibolite-facies metasediments from Greenland and northern Labrador studied here belong to the Isua greenstone belt (IGB)¹⁴ and to the Nulliak supracrustal sequence¹⁵, respectively, and were probably deposited during the waning phase of the LHB. The sedimentary precursors of the samples are interpreted as erosion products of mainly mafic rocks (see Supplementary Information). Four of the six early Archaean metasediment samples have resolvably less radiogenic W than the accessible Earth, with ϵ_{W} values ranging from -0.44 to -1.23 (Table 1, Fig. 1). All four data points were replicated on separate aliquots (6X, 4X, 2X, 2X). One sample (KC/91/55c), with large anomaly (-1.09 ± 1.04 ϵ_{W}) but also large uncertainty, was not replicated owing to insufficient W yield. Tungsten in a further sample is indistinguishable from the terrestrial average ($+0.07 \pm 0.46$ ϵ_{W}). Importantly, because the stable isotope ratios ($^{183}\text{W}/^{186}\text{W}$ and

$^{184}\text{W}/^{186}\text{W}$) of all samples were exactly reproduced to the ACQUIRE standard values (see Supplementary Information), these ^{182}W anomalies are real, and cannot be attributed to spectral interference on a stable W isotope used for mass bias correction. As shown previously¹², the existence of a W isotope anomaly can be corroborated by study of the stable isotope ratio data. To our knowledge, these are the first terrestrial samples shown to contain W isotope anomalies.

Two observations are of particular importance for interpreting this W isotope array. First, an early Archaean terrestrial W crustal average sample (prepared by homogenizing equal aliquots of 24 samples of ~ 3.8 -Gyr igneous felsic and mafic gneisses¹⁶) yields the terrestrial W isotope ratio (-0.12 ϵ_{W} ; Table 1), implying that unradiogenic W was not characteristic of the early Archaean silicate Earth. Hence the most plausible explanation for the unradiogenic W isotope compositions is that a proportion of the W in the metasediments is of extraterrestrial origin. Second, there is strong curved covariation between W isotope composition and several trace element ratios in the sample suite (Fig. 2). The highest correlation coefficients are obtained for logarithmic fits between W isotope compositions and Cr/Ti (Fig. 2) and Ni/Nb ratios. This implies that unradiogenic W was introduced from an erosion source rich in compatible elements. Both chondrites and iron meteorites are rich in Ni and Cr, and have unradiogenic W. Indeed, the logarithmic fit through the metasediment data (Fig. 2a) extrapolates to chondrite and iron meteorite data points.

This does not mean that the metasediments are impact ejecta composed of mixed terrestrial and meteoritic debris. It is more plausible that when these rocks were deposited as water-lain sediments they were fed from weathered terrestrial rock from the eroding land surface, and from meteorite debris that had collected on that surface during the LHB, because the metasediment data do

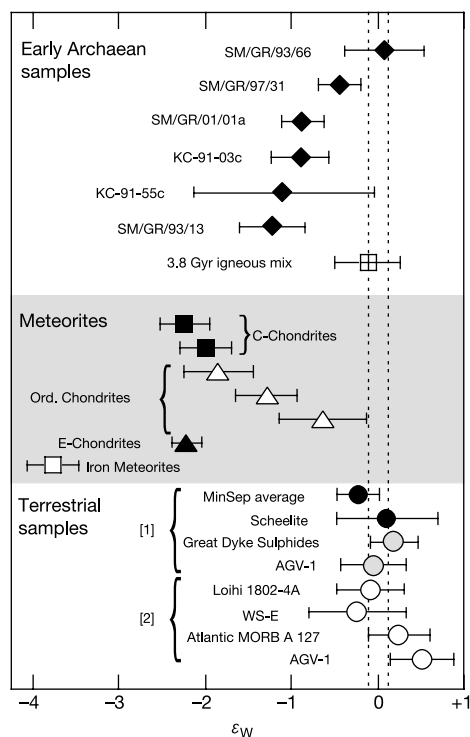


Figure 1 Values of ϵ_{W} for terrestrial rocks, meteorites and early Archaean samples relative to the composition of a tungsten standard, ACQUIRE-W. Dashed lines show 2 s.d. of mean uncertainty envelope of the standard ($n = 46$). Grey circles, terrestrial samples measured previously⁷; filled circles, present results (Table 1). Results highlighted with [1] were analysed at the University of Queensland. Open circles, previously published^{8,21} terrestrial samples. Results highlighted with [2] were analysed at the University of Michigan. Open square, average of published iron meteorites⁹; filled triangle, average of bulk rock enstatite chondrites¹⁰; open triangles, bulk rock ordinary chondrites¹²; filled squares, new data for Allende carbonaceous chondrite^{7,13}. Crossed square, average early Archaean crust (text and Table 1); filled diamonds, early Archaean metasediments (text and Table 1). External error (22 p.p.m.) was assigned to samples with smaller internal errors.

Table 1 Tungsten isotope data

Sample	$^{182}\text{W}/^{183}\text{W}$	$\pm$	ϵ_{W}	$\pm$
Terrestrial samples				
Mean ACQUIRE-W	1.852512	19	+0.00	0.10
Scheelite A	1.852450	15	-0.33	0.08
Scheelite B	1.852545	22	+0.18	0.12
Scheelite C	1.852571	19	+0.32	0.10
Scheelite D	1.852556	33	+0.24	0.18
Average	1.852531	109	+0.10	0.59
MinSep average	1.852470	22	-0.22	0.12
Early Archaean samples—Greenland				
3.8 Gyr igneous mix	1.852490	70	-0.12	0.38
SM/GR/93/13 A	1.852258	89	-1.37	0.48
SM/GR/93/13 B	1.852281	93	-1.25	0.50
SM/GR/93/13 C	1.852308	33	-1.10	0.18
SM/GR/93/13 D	1.852306	30	-1.11	0.16
SM/GR/93/13 E	1.852230	33	-1.52	0.18
SM/GR/93/13 F	1.852325	37	-1.01	0.20
Average	1.852284	71	-1.23	0.38
SM/GR/01/01a A	1.852339	122	-0.93	0.66
SM/GR/01/01a B	1.852356	44	-0.84	0.24
Average	1.852348	24	-0.89	0.13
SM/GR/97/31 A	1.852427	104	-0.45	0.56
SM/GR/97/31 B	1.852431	63	-0.43	0.34
Average	1.852429	6	-0.44	0.03
SM/GR/93/66	1.852525	85	+0.07	0.46
Early Archaean samples—Labrador				
KC-91-55c	1.852309	193	-1.09	1.04
KC-91-03c A	1.852355	41	-0.85	0.22
KC-91-03c B	1.852343	74	-0.91	0.40
KC-91-03c C	1.852304	30	-1.12	0.16
KC-91-03c D	1.852379	26	-0.72	0.14
Average	1.852345	62	-0.90	0.34

All measurements were obtained using our previously outlined procedure⁷, except that samples were treated with aqua regia before W purification. Replicate analysis represent separately digested aliquots. All uncertainties are given at 95% confidence. Samples: ACQUIRE-W standard prepared from a high purity W ribbon ($n = 46$); Scheelite is a museum specimen from the late Archaean eastern Yilgarn craton, Australia; MinSep average contains surplus mineral separates from metamorphic and igneous rocks ranging from 0.4 to 3.8 Gyr; 3.8-Gyr igneous mix consists of equal aliquots of 17 felsic and 7 mafic ~ 3.8 -Gyr gneisses from south of the IGB¹⁶.

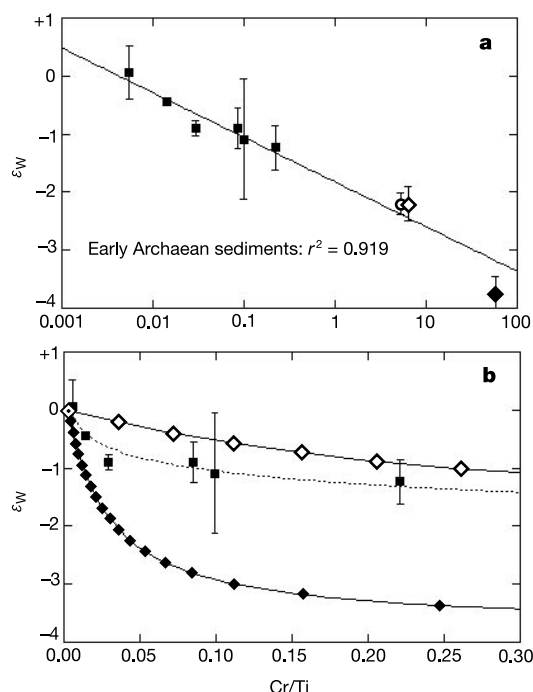


Figure 2 Comparison of ϵ_W to Cr/Ti ratio. **a**, Covariation between ϵ_W and Cr/Ti ratio (note that the x axis is on a logarithmic scale). All metasediment data points from this study (filled squares) define a tight ($r^2 = 0.919$) logarithmic fit (solid line). The fit extends to average enstatite chondrites¹⁰ (open circle), the carbonaceous chondrite Allende^{7,13} (open diamond), and close to average iron meteorite⁹ (solid diamond). Elemental abundance data from refs 17 and 22. Iron meteorites were assigned a Ti abundance of 2 p.p.m., based on the observation¹⁷ that troilite inclusions contain about 10 p.p.m. Ti. **b**, Comparison of metasediment data (solid squares with error bars connected by dashed line representing logarithmic fit) with model mixing hyperbolae. One hyperbola (open diamonds) was calculated between a hypothetical chondrite endmember (data sources as in **a**) and a terrestrial endmember using Cr and Ti concentration data from the IGB metapelite with the lowest known Cr/Ti ratio (0.002) and the lowest observed W content of 100 p.p.b. This endmember represents the Hadean crust, and was assigned an ϵ_W of zero. The second hyperbola (filled diamonds) was calculated between a hypothetical iron meteorite endmember (data sources as in **a**) and the terrestrial endmember. In this case, the terrestrial endmember was assigned a relatively high W content of 3,000 p.p.b.

not show the appropriate hyperbolic relationship (Fig. 2b) predicted by mixing of unmodified terrestrial and meteorite debris. We propose that weathering of meteoritic debris caused preferential liberation of certain elements, depending on the stability of the host minerals in the Hadean atmosphere and hydrosphere. For example, most Cr in iron-meteorite is hosted by troilite (FeS), whereas all W is found in the FeNi metal phase¹⁷. Although W isotopes cannot be used to directly identify the nature of the meteoritic impactors, our data nevertheless provide evidence for the oldest impact event(s) so far discovered on Earth, lending support to interpretation of slightly increased Ir concentrations in some IGB lithologies¹⁸.

Most of the studied metasediments contain particles of carbon. In particular, sample SM/GR/01/01a is from the same outcrop where Rosing¹⁹ described discrete graphite microparticles with isotopically light C ($\delta^{13}\text{C}$ approximately -19‰), which he interpreted as biogenic. However, in view of the present evidence for extraterrestrial W in this sample, the possibility needs to be considered that the graphite represents insoluble carbon particles from carbonaceous chondrites, with $\delta^{13}\text{C}$ of about -18‰ (ref. 20). □

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Competing interests statement

The authors declare that they have no competing financial interests.

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A long-tailed, seed-eating bird from the Early Cretaceous of China

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The lacustrine deposits of the Yixian and Jiufotang Formations in the Early Cretaceous Jehol Group in the western Liaoning area of northeast China are well known for preserving feathered dinosaurs, primitive birds and mammals^{1–3}. Here we report a large basal bird, *Jeholornis prima* gen. et sp. nov., from the Jiufotang

Formation. This bird is distinctively different from other known birds of the Early Cretaceous period in retaining a long skeletal tail with unexpected elongated prezygopophyses and chevrons, resembling that of dromaeosaurids^{4–6}, providing a further link between birds and non-avian theropods^{7–8}. Despite its basal position in early avian evolution, the advanced features of the pectoral girdle and the carpal trochlea of the carpometacarpus of *Jeholornis* indicate the capability of powerful flight. The dozens of beautifully preserved ovules of unknown plant taxa in the stomach represents direct evidence for seed-eating adaptation in birds of the Mesozoic era.

Aves L., 1758

Jeholornis prima gen. et sp. nov.

Holotype. IVPP (Institute of Vertebrate Paleontology and Paleoanthropology, Beijing, China) collection number V13274, a nearly completely articulated skeleton.

Etymology. The generic name is derived from the holotype-bearing

'Jehol Group', which contains the Jehol biota. The specific name refers to its primitive appearance in the tail.

Locality and horizon. Dapingfang, Chaoyang City, western Liaoning, China; Jiufotang Formation, middle Early Cretaceous. Associated vertebrate fossils include feathered dromaeosaurs⁹ and several primitive birds such as the ornithurine *Yanornis*¹⁰, *Confuciusornis*², the newly reported *Sapeornis*¹¹ and abundant enantiornithines¹².

Diagnosis. A large bird with the following derived characters: lachrymal with two vertical and elongated pneumatic fossae; mandibles robust with well ossified symphysis; first phalanx of the third manual digit twice as long as the second phalanx, which together form a bow-shaped structure; 20 caudal vertebrae behind the transition point; lateral trabecula of the sternum with a rounded fenestra at the distal end; ratio of forelimb (humerus plus ulna plus carpometacarpus) to hindlimb (femur plus tibiotarsus plus tarsometatarsus) of about 1.2.

Description. *Jeholornis* is a large bird, represented by a partially articulated skull and nearly complete postcranial bones (Fig. 1). The holotype comprises five slabs. Their associations are unambigu-

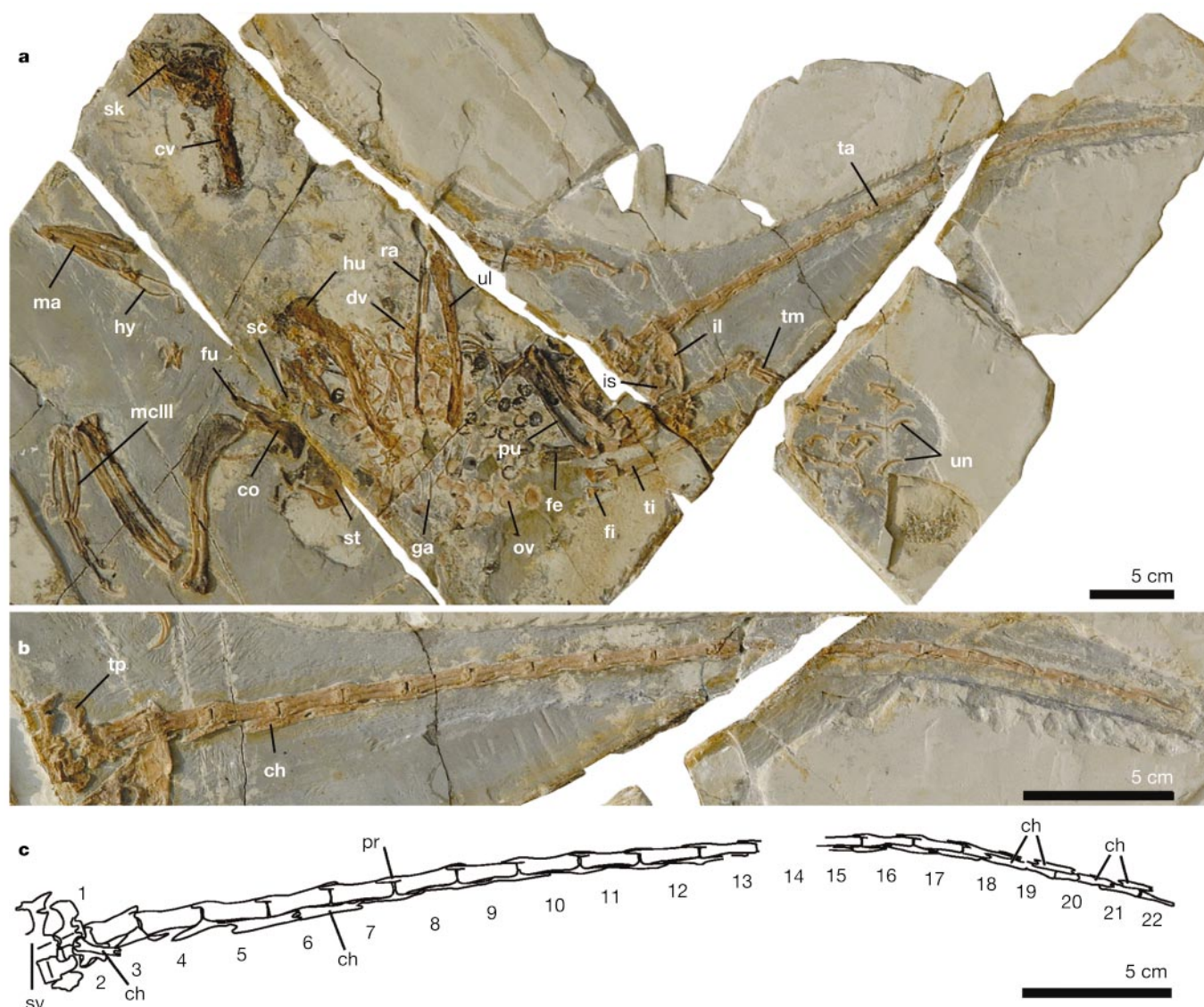


Figure 1 Complete holotype of *Jeholornis prima* gen. et sp. nov. (IVPP V13274).

a, Skeleton. **b**, Caudal vertebrae. **c**, Line drawing of the caudal vertebrae. ch, chevron; co, coracoid; cv, cervical vertebra; dv, dorsal vertebra; fe, femur; fi, fibula; fu, furcula; ga, gastralia; hu, humerus; hy, hyoid bone; il, ilium; is, ischium; ma, mandible; mcIII,

metacarpal III; ov, ovule; pr, prezygopophysis; pu, pubis; ra, radius; sc, scapula; sk, skull; st, sternum; sv, sacral vertebra; ta, tail; ti, tibia; tm, tarsometatarsus; tp, transverse process; ul, ulna; un, unguals; 1–22, caudal vertebrae 1–22.

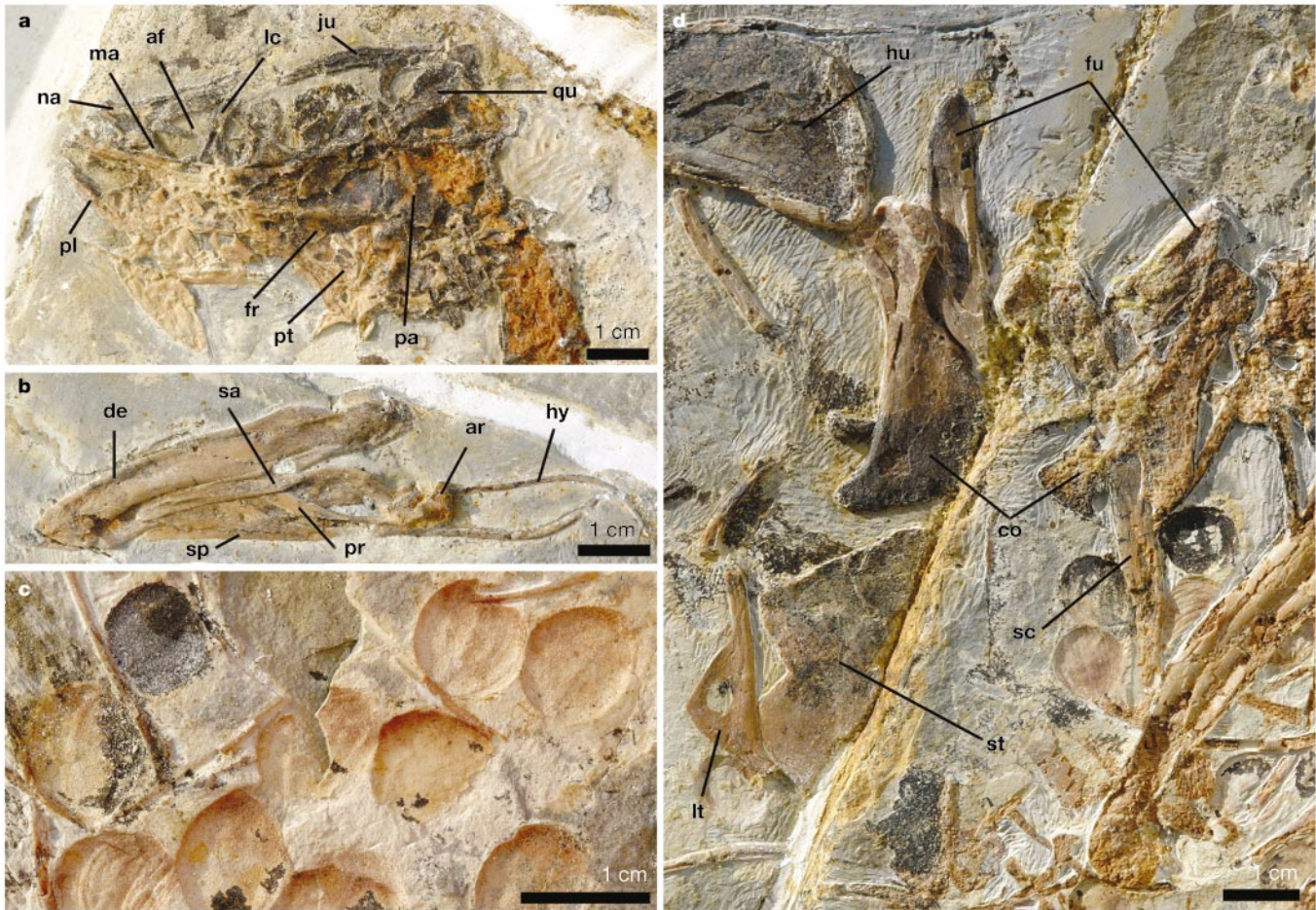


Figure 2 Holotype of *Jeholornis prima* gen. et sp. nov. (IVPP V13274). **a**, Skull. **b**, Mandibles. **c**, Ovules. **d**, Pectoral girdle and sternum. af, antorbital fenestra; ar, articular; de, dentary; fr, frontal; ju, jugal; lc, lachrymal; lt, lateral trabecula of the sternum; ma, maxilla; na, nasal; pa, parietal; pl, palatine; pr, prearticular; pt, pterygoid; qu, quadrate; sa, surangular; sp, splenial. See Fig. 1 for other abbreviations.

ously supported by, among other evidence, the presence of at least one skeletal element on two neighbouring slabs. For example, the association of the two principal slabs—that is, one slab with the skull and the other slab containing the most caudals—is supported by the distribution of a pubis, an ischium and two tibiae on both slabs. Furthermore, all of the slabs that include parts comprising the whole tail have been prepared completely by a professional technician under our supervision in the laboratory. Therefore, the possibility of a composite specimen for the holotype can be ruled out.

The maxilla is reduced and does not bear any teeth. There is a large antorbital fenestra. The lachrymal is 'T'-shaped with two elongated pneumatic fossae vertically distributed (Fig. 2a). The jugal is rod-shaped; it has a long, slender and posteriorly curved postorbital process similar to that of *Sinornithosaurus*¹³. The isolated mandibles are robust and they are well fused at the rostral end.

There are three very small conical teeth on the left mandible. Of note, the teeth of the Late Cretaceous *Gobipteryx* are also reduced together with the development of a fused mandibular symphysis. Two well-developed hyoid bones are long, slender and curved (Fig. 2b).

The cervical vertebrae are robust, and there are at least ten cervicals. There are 22 caudals that are nearly completely articulated in preservation. The last sacral has an expanded transverse process at the distal end and it is articulated with the first caudal. The two proximal caudals are short, with well-developed transverse processes. The second caudal has a rod-shaped chevron, with a forked caudal end. There are 20 elongated caudal vertebrae behind the transition point, with distinctively elongated prezygopophyses and chevrons (Fig. 1b, c). The prezygopophyses of the posterior caudal vertebrae extend to more than one-third the length of the preceding caudals. The horizontally distributed chevrons of the last 20 caudals

Table 1 Comparison of *J. prima* gen. et sp. nov. (IVPP V13274) with other primitive birds and theropods

Selected element	<i>Jeholornis prima</i> (IVPP V13274)	<i>Sapeornis chaoyangensis</i> (IVPP V12698)	<i>Archaeopteryx bavarica</i> (Solnhofen specimen)	<i>Confuciusornis sanctus</i> (IVPP V11619)	<i>Sinornithosaurus millenii</i> (IVPP V12811)	<i>Microraptor zhaoianus</i> (IVPP V12330)
Humerus	110 (r)	127 (l)	83	52 (r)	134 (r)	—
Ulna	109 (r)	133 (l)	72*	47 (r)	110 (r)	35 (r)
Metacarpal II	47 (r)	57 (l)	34†	27 (r)	63 (r)	—
Pubis	64 (r)	85 (l)	59	47 (r)	116 (l)	—
Femur	75 (r)	80 (l)	70*	47 (l)	148* (l)	53 (l)
Tibiotarsus/tibia	88 (r)	83 (l)	90	54 (l)	—	68 (l)
Metatarsal III	47 (r)	44 (l)	48	25 (l)	93	34 (l)

Measurements are in millimetres. l, left side; r, right side.

* Estimated values.

† Preserved length.

are all connected at their forked extremities. The last caudal tapers distally and is medio-laterally compressed. The gastralia are long, slender and rod-like.

The scapula and coracoid are not fused. The scapula is curved and tapers caudally, as in advanced birds. The coracoid is strut-like, with a well-developed lateral process. It is more elongated than in both *Archaeopteryx*^{14,15} and *Sapeornis*, but is shorter and more robust than in more advanced birds such as enantiornithines¹⁶. Most of the medial margin of the coracoid is convex as in *Archaeopteryx* and *Confuciusornis*^{17,18}. The furcula is robust, shaped like a boomerang, and is generally similar to those in *Archaeopteryx*, *Confuciusornis* and some dromaeosaurids. The sternum is short; the lateral trabecula is unfused with the main body of the sternum, with a rounded fenestra near the caudal end (Fig. 2d).

The ratio of the forelimb (humerus plus ulna plus carpometacarpus) to hindlimb (femur plus tibiotarsus plus tarsometatarsus) is about 1.2, which is much larger than in *Archaeopteryx* (less than 1); however, *Sapeornis*¹¹ and the enantiornithine *Longipteryx*¹² have forelimbs that are relatively longer among early birds (Table 1).

The humerus has a large deltoid crest, and the ventral tubercle is not well developed. The radius is slightly shorter than the humerus, as in *Archaeopteryx* and *Confuciusornis*. The manus nearly equals the humerus and ulna in length (Fig. 1a). The carpometacarpus is fused at the proximal end, with a well-developed carpal trochlea. The third metacarpal is bow-shaped and is tightly attached to the second metacarpal at the distal end. The ulna has a well-developed metacarpal incision. There are three large and curved unguals in the hand. Unlike *Archaeopteryx*, *Confuciusornis* and *Protopteryx*¹⁹, the first phalanx of the first digit does not extend to the distal end of the second metacarpal, which is similar to that of *Sapeornis*¹¹ and more advanced enantiornithines and ornithurine birds¹⁰. The third digit comprises four phalanges as in *Archaeopteryx* and *Confuciusornis*. The second phalanx of the third digit is less than half the size of the first phalanx. It is noteworthy that new materials of *Sapeornis* show that it has only two reduced phalanges, contrary to previous reconstruction.

The pelvis is most similar to that of *Archaeopteryx* with respect to the position of the pubis relative to the ilium; the pubes are less caudally retroverted than in some dromaeosaurs²⁰. The pubic symphysis appears to be short. The pubic foot is spoon-shaped as in *Archaeopteryx* and some enantiornithines, but is different from

that of *Confuciusornis*. The ischium has a marked strut-like proximal dorsal process as in *Sapeornis*, *Confuciusornis* and enantiornithines (Fig. 3a). A less-well-developed process is present in *Archaeopteryx* and non-avian theropods such as *Sinornithosaurus* and *Unenlagia*²¹.

The femur has a deep and narrow popliteal fossa at the distal end. The ankle is generally similar to that of *Archaeopteryx*, *Rahonavis*²² and non-avian theropods such as *Sinornithosaurus* in having an unfused calcaneum and an astragalus with an ascending process (Fig. 3b–e). The calcaneum is narrow, nearly rounded, and about one-fifth the width of the astragalus. It seems that the character of the astragalus (that is, a large ascending process) of non-avian theropods is hardly modified in the most basal birds, and the pretibial bone is most probably a new trait that only appeared in a later stage of avian evolution²³.

The tarsometatarsus is fused at the proximal end, as in all known birds. The fifth metatarsal is present as in *Archaeopteryx*, *Confuciusornis* and *Sapeornis*. The hallux is reversed as in all birds, but is unknown in any non-avian theropods. The unguals of the foot are large and curved, as in most basal birds. The hypertrophied second ungual is similar to that of *Archaeopteryx* and *Rahonavis*²², and is also reminiscent of the situation of dromaeosaurids and troodontids^{6,20,22}. The second phalanx of the second digit is longer than the first phalanx, as in *Microraptor* and nearly all basal birds.

Although feathers have been found to be associated with various birds and dromaeosaurs from the same locality, they have not been preserved with the holotype of *Jeholornis*.

In the stomach position of *Jeholornis*, over 50 ovules are preserved (Fig. 1a). These are mainly rounded and average 8–10 mm in width and length (Fig. 2c). Similar ovules have been reported by palaeobotanists elsewhere, and are referred to the genus *Carpolithus*; however, they belong to an unknown plant group²⁴.

Except for *Sapeornis*, which has a short tail and a pygostyle, and derives from the same area and horizon, *Jeholornis* is the largest bird known from the Early Cretaceous. Both genera are also larger than the Late Jurassic *Archaeopteryx* (Table 1). Except for *Archaeopteryx* and *Rahonavis*, *Jeholornis* is the only known bird with a long caudal series (Fig. 1). The tail is longer than the hindlimb; however, in the largest *Archaeopteryx*, the tail is shorter than the hindlimb¹⁵. The chevrons are also better developed and more elongated in *Jeholornis* than in *Archaeopteryx*. There are 20 caudals behind the transition point in *Jeholornis*, and there are less than 20 in *Archaeopteryx*^{15,25}. The presence of such a primitive skeletal tail largely resembling that of dromaeosaurids provides further evidence supporting the relationship between birds and dromaeosaurids (Fig. 4). The

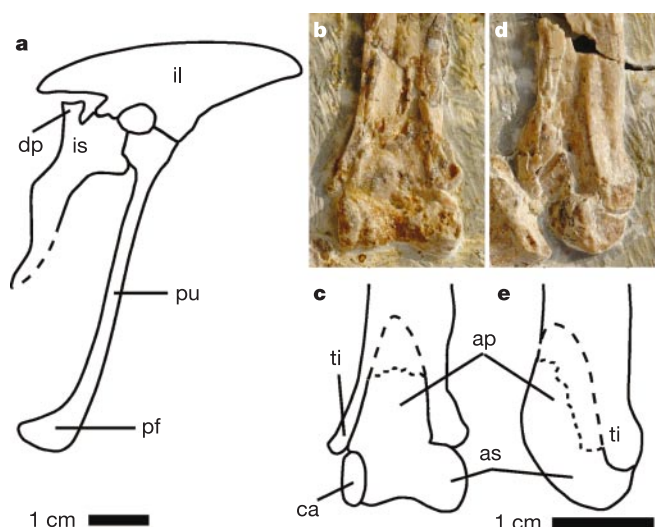


Figure 3 Pelvic girdle and tibia, calcaneum and astragalus of *Jeholornis prima* gen. et sp. nov. (IVPP V13274). **a**, Reconstruction of the pelvic girdle (right) in lateral view. **b–e**, Distal end of the tibia and its relationship with the calcaneum and astragalus (**b**, **c**, right, in cranial view; **d**, **e**, left, in cranio-lateral view). ap, ascending process of the astragalus; as, astragalus; ca, calcaneum; dp, dorsal process of the ischium; pf, pubic foot. See Fig. 1 for other abbreviations.

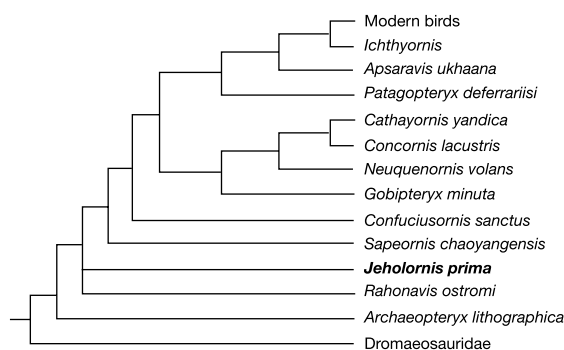


Figure 4 Cladogram showing phylogenetic relationships between *Jeholornis prima* and other major groups of birds. We used the PAUP 4.0 beta 10 method for phylogenetic analysis. We followed the same method as in ref. 28 by using 201 characters and analysing 18 taxa, with Dromaeosauridae as an outgroup. We also revised the data matrix from ref. 28. Four most parsimonious trees were obtained. Consistency index = 0.72; retention index = 0.83; tree length = 339. The cladogram is simplified from the congruent tree (see Supplementary Information).

derived features of the pectoral girdle of *Jeholornis* such as a strut-like coracoid and the well-developed carpal trochlea of the carpo-metacarpus, suggest the capability of powerful flight.

One of the most significant features of *Jeholornis* is the preservation of dozens of ovules in the stomach (Fig. 2c). Although hundreds of excellently preserved Mesozoic birds such as *Confuciusornis* have been discovered, our knowledge about their diet has been at best speculative. *Jeholornis* represents direct evidence for seed-eating adaptations in Mesozoic birds. The ovules, referable to the generic name *Carpolithus*²⁴, cannot be positively included into any of the chief plant groups (J. Hilton and Q. Leng, personal communication). It is difficult to determine whether, in life, *Jeholornis* ate cones on a tree, ovules from intact cones, or ovules shed from their cones. The intact nature of the ovules, however, may indicate that the bird ate them whole, to be digested in the gizzard, rather than breaking them up to eat them in small pieces (J. Hilton, personal communication). The large number of seemingly undigested ovules in the specimen probably indicates a large crop. Furthermore, the robust mandibles with fused mandibular symphysis, reduced teeth and well-developed hyoid bones seem to lend further support for the seed-eating habit of *Jeholornis*.

Jeholornis certainly possessed an arboreal capability, as evidenced by its reversed hallux, long and strongly curved pedal unguals, and toe proportions (Fig. 1a). However, as in other basal birds such as *Archaeopteryx* and *Confuciusornis*, there is no evidence to discount the possibility that *Jeholornis* spent some time on the ground^{26,27}. Therefore, without further evidence, it is difficult to conclude whether *Jeholornis* fed on ovules from cones on trees, or on the ground. This discovery, together with many others in recent years, suggests that by the Early Cretaceous, early birds had not only diverged significantly in morphology, size and ecology^{11,28}, but had also differentiated with respect to feeding adaptation. □

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Mechanisms of long-distance dispersal of seeds by wind

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Long-distance dispersal (LDD) is central to species expansion following climate change, re-colonization of disturbed areas and control of pests^{1–8}. The current paradigm is that the frequency and spatial extent of LDD events are extremely difficult to predict^{9–12}. Here we show that mechanistic models coupling seed release and aerodynamics with turbulent transport processes provide accurate probabilistic descriptions of LDD of seeds by wind. The proposed model reliably predicts the vertical distribution of dispersed seeds of five tree species observed along a 45-m high tower in an eastern US deciduous forest. Simulations show that uplifting above the forest canopy is necessary and sufficient for LDD, hence, they provide the means to define LDD quantitatively rather than arbitrarily. Seed uplifting probability thus sets an upper bound on the probability of long-distance colonization. Uplifted yellow poplar seeds are on average lighter than seeds at the forest floor, but also include the heaviest seeds. Because uplifting probabilities are appreciable (as much as 1–5%), and tree seed crops are commonly massive, some LDD events will establish individuals that can critically affect plant dynamics on large scales.

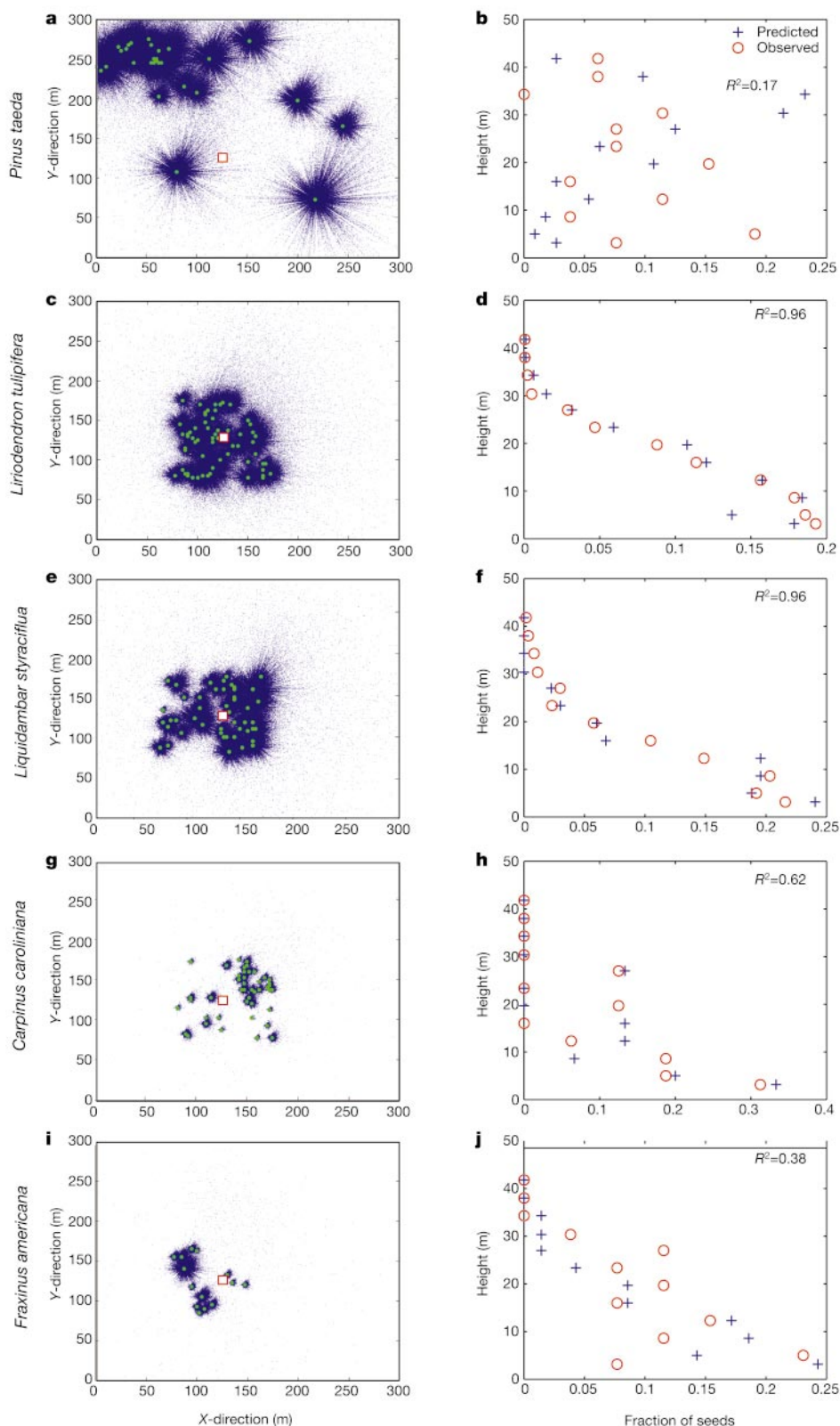


Figure 1 Seed dispersal simulations of five wind-dispersed tree species around the Duke Forest tower, for 35 days during the autumn of 2000. **a, c, e, g, i**, The difference in spatial distribution among species—together with differences in dispersal factors (Table 1)—generate distinct seed shadow (left column; green circles represent adult trees and the red square indicates the tower). **b, d, f, h, j**, Vertical profiles of predicted proportions of seeds at different heights along the tower generally match the observed profiles (right column). The fits are high for the two species for which the observed profiles are estimated from large

samples (Table 1); for the other species, the coefficient of determination (R^2) was lower. Nonetheless, the low R^2 is associated with weak vertical gradients in seed proportions (compare **b** and **d**) which appear random (or well mixed), in particular in **b**, where both observed and predicted distribution describe a random distribution (Runs up-and-down randomness test, $P > 0.1$). In such cases, the model correctly predicts a random vertical distribution, albeit at a low R^2 . We emphasize that R^2 is not an appropriate model diagnostic here as R^2 is inherently low between two approximately random distributions.

Table 1 Canopy and seed aerodynamic attributes for dispersal simulations

Tree species	Seed terminal velocity (m s^{-1} ; mean $\pm$ s.d.)	Tree height (m; mean $\pm$ s.d.)	Seed release height (fraction of tree height; mean)	Seed counts in traps	Estimated uplifting probability (%)	
					From trap data	From simulations
<i>Pinus taeda</i>	0.70 ± 0.13	31.49 ± 3.61	0.72	36	11.11	4.80
<i>Liriodendron tulipifera</i>	1.48 ± 0.52	26.13 ± 7.82	0.66	3,294	0.36	1.80
<i>Liquidambar styraciflua</i>	1.05 ± 0.24	25.55 ± 7.04	0.63	1,609	1.42	0.98
<i>Carpinus caroliniana</i>	0.98 ± 0.21	11.17 ± 2.78	0.74	17	0	0.05
<i>Fraxinus americana</i>	1.41 ± 0.26	18.72 ± 5.91	0.70	33	0	0.02

The two main biological parameters are terminal velocity of seeds and height of seed release (see Methods for empirical estimation). The height of seed release is the product of the height of reproductive trees and the fraction from tree height at which seeds are released. Predicted seed uplifting probabilities, based on simulations of 10^5 to 10^8 seeds for each species, are compared with direct estimates based on seed-trap data collected in the autumn of 2000 along a 45-m high tower in Duke Forest. The total number of seeds counted in all traps during this period is also given.

Many common and economically important tree species, especially those of temperate and boreal forests, have morphological adaptations that facilitate dispersal by wind^{13,14}. It has long been suggested that wind updrafts provide the key mechanism for LDD of seeds^{13,15,16}. However, while uplifting of parachute or fluffy seeds is commonly observed, seeds of many wind-dispersed tree species are relatively heavy, and fall in still air with terminal velocities typically greater than the usual vertical wind velocity. Uplifting events, in which upward vertical wind velocity exceeds seed terminal velocity, are rare and difficult to quantify. Consequently, despite successful predictions of the short-distance dispersal of most seeds by both phenomenological^{5,17,18} and mechanistic^{19,20} models, attempts to predict rare events of LDD have repeatedly failed^{9–12}. Phenomenological models are fitted to the observed data, which are limited by inherent difficulties in obtaining data on LDD^{6–8}, and extrapolation beyond the observed range is questionable. The failure of previous mechanistic models probably emanates from their use of time-averaged wind statistics (for example, hourly and longer), thereby averaging over the critically strong turbulent updrafts with timescales of the order of seconds. Here, we propose a new generation of mechanistic dispersal models based on the intermittent yet coherent nature of canopy turbulent airflow within vegetation. We show that for species representing a broad spectrum of seed and tree morphologies, such models can reliably predict important statistical attributes of rare LDD events.

We applied a coupled eulerian–lagrangian approach^{21,22} to describe the turbulent statistics within and above the canopy (see Methods). All parameters were independently estimated from

laboratory and field experiments. The model was tested against further wind and seed data collected along a 45-m walk-up tower in a 33-m tall, 80-yr-old deciduous stand in Duke Forest, North Carolina ($35^\circ 58' \text{ N}$, $79^\circ 08' \text{ W}$; see Methods). Velocity statistics estimated by the moment closure model agreed well with the high frequency wind data measured at three heights along the tower. This agreement is consistent with earlier comparisons²³.

We then tested the coupled eulerian–lagrangian model against seed data collected in 102 traps placed at 12 levels along the tower. The model simulated the three-dimensional dispersal of seeds given: (1) location of source trees around the tower, (2) seed terminal velocity and (3) distribution of seed release height (Table 1, Fig. 1; and see Methods for simulation details). Observed proportions of seeds at different heights along the tower closely match the model's predictions (Fig. 1).

During a period of 35 days in the autumn of 2000, we collected 4,989 seeds of five wind-dispersed tree species in the traps along the tower. Thirty-nine seeds belonging to three species were collected above the top of the canopy (Table 1). Direct calculation of seed uplifting probability from the observed proportion of seeds above the top-canopy height at a single tower is problematic. This proportion depends on the specific spatial arrangement of the trees around the tower. At our site, the observed proportion would underestimate true uplifting probability for species clumped close to the tower, such as yellow poplar (*Liriodendron tulipifera*) (Fig. 1c), since some seeds caught in vertical updrafts may terminate their flight in traps before escaping the canopy. For species far from the tower, such as loblolly pine (*Pinus taeda*) (Fig. 1a), the observed proportion would overestimate the true uplifting probability, since only seeds travelling relatively long distances are trapped at the tower, and these are more likely to have been uplifted.

The above sampling problem could be resolved by placing seed-traps along many side-by-side towers. Because such a set-up is intractable in tall forests, we estimate uplifting probabilities using the verified dispersal model. Given that the model reliably describes the vertical structure of both wind statistics and trapped seeds, its predictions of uplifting probabilities can be used to provide a spatially integrated estimate of the number of seeds of each species arriving at each height on the tower. Comparisons between these two estimates (Table 1) reveal that the model corrected direct estimates in the expected direction; that is, it increased the underestimated probability for yellow poplar, and reduced the overestimated probability for loblolly pine.

We next used the verified model to estimate dispersal kernels of wind-dispersed tree species, assuming that a model that predicts the frequency of LDD could also provide reasonable estimates for travel distances. However, seeds travelling long distances are likely to encounter variable wind conditions during flight, which may be different from those at the site of release. We therefore ran two sets of simulations, one with a planar, homogeneous wind, the other with variable wind (see Methods for simulation details). In both cases, we found that dispersal kernels were bimodal: seeds that were uplifted travelled much further than those that did not escape the forest canopy (Fig. 2). Most seeds are not uplifted and are predicted to travel only up to several hundred metres, with a modal distance of

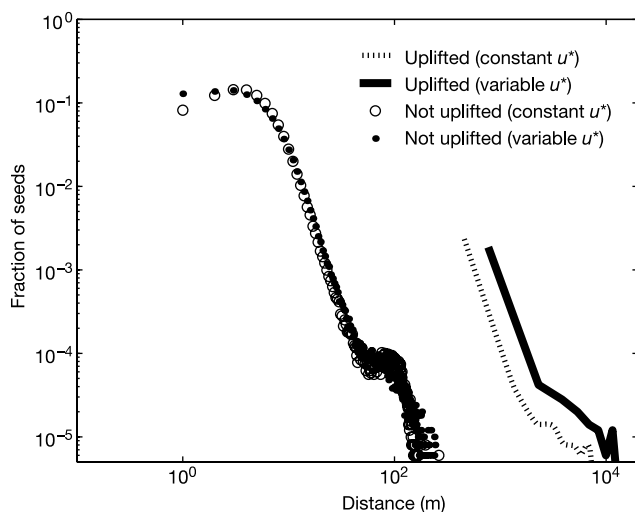


Figure 2 Bimodal dispersal kernel for yellow poplar (*Liriodendron tulipifera*) seeds released from 18 m in a 33-m-high forest, with a secondary peak at the tail generated exclusively by seed uplifting. The same pattern emerges assuming either constant or variable friction velocity (u^*), of 0.8 and $0.8 \pm 0.4 \text{ m s}^{-1}$, respectively. Each dispersal kernel is computed from simulations of 500,000 dispersal events.

roughly the canopy height. In stark contrast, the few seeds that are uplifted are predicted to travel at least several hundred metres, and perhaps tens of kilometres. These findings hold for both spatially constant and variable wind conditions. We conclude that the seed uplifting probability, or equivalently the frequency of LDD, is predictable from the statistical distributions of seed release height, seed terminal velocity, and turbulent flow at the time of release. A consequence of such a clearly bimodal dispersal kernel (Fig. 2) is that quantitative rather than arbitrary distinction between short- and long-distance dispersal is possible for wind-dispersed tree species^{6,8}.

While standard phenomenological models assume a gradual monotonic decline of the frequency of dispersal events with distance^{5,17,18}, our mechanistic model predicts a bimodal distribution of short- and long-distance dispersal²³ (Fig. 2). Such bimodal distributions mirror the two flow regimes: (1) short-distance, controlled by within-canopy flow and characterized by small mean windspeed, damped turbulence, and energetic eddies of approximately one-third of the mean canopy height²⁴, and (2)

long-distance, controlled by atmospheric surface-layer turbulence having mean windspeed and characteristic eddy sizes that rapidly increase with increasing height above ground.

Seeds uplifted and dispersed long distances are meaningful for plant dynamics only if establishment follows dispersal. Thus, developing a predictive ability for LDD—as shown here—is necessary but not sufficient in developing predictive ability for long-distance plant colonization. Information on two other major processes—fecundity and post-dispersal survival—is needed. Fecundity in wind-dispersed trees is usually high, for example, on the order of 10^4 seeds per tree per year for yellow poplar²⁵, but varies considerably among species, sites, individuals and over time¹⁸. Mortality occurs mostly during seed and seedling stages⁷, and seed-to-sapling survival probability also varies considerably among species and sites. For example, in two sites close to ours, survival has been estimated as 6% for loblolly pine, and 0% in one site and 0.06% in another for yellow poplar²⁶. The survival of seeds travelling long distances is poorly quantified, because LDD events are rare and difficult to track; quantifying this survival probability is a major current challenge²⁷. Seeds dispersing very far may have higher survival probabilities than seeds dispersing near, because they escape the high mortality caused by density-dependent processes such as competition, predation and disease. Furthermore, depending on the pattern of spatial autocorrelation in the environment, they may be deposited at sites that are more (or less) suitable for establishment than sites near the source location⁷.

Post-dispersal survival depends not only on the environment but also on seed traits. Lighter seeds are expected to fall more slowly, hence are more likely to be uplifted and dispersed further, but they also make less competitive seedlings. We tested such differences by comparing the mass and terminal velocity of yellow poplar seeds collected at traps above the canopy and, during the same period, at the forest floor (adequate samples were available only for this species). We found no difference in terminal velocity (Fig. 3a; $P > 0.5$), in accordance with an independent test we carried out in Princeton, New Jersey²⁸. However, seeds collected above the canopy were significantly lighter than those collected at the forest floor (Fig. 3b; $P = 0.004$). This apparent discrepancy is explained by the fact that the relatively light yellow poplar seeds that are uplifted have relatively small surface area (Fig. 3c; $P = 0.003$), hence, their terminal velocity is conserved. Despite the statistical difference between their means, the two sample distributions overlap extensively and samples appear to come from the same population (Fig. 3). This suggests that relatively heavy yellow poplar seeds also get uplifted, so the overall effect of the difference in mean seed mass between uplifted and non-uplifted seeds on establishment is not expected to be strong.

There is still a high degree of uncertainty in predicting long-distance colonization. Probabilities of LDD, as quantified here, can be as large as 1–5%, hence making long-distance colonization more frequent than previously believed. Nevertheless, we found that for some species, uplifted seeds are on average lighter than non-uplifted seeds, suggesting they are less likely to germinate and survive seedling competition, making long-distance colonization more difficult. Multiplying the arrival and survival probabilities by the typical high fecundity of wind-dispersed trees ($\sim 10^4$ seeds per tree per year) should yield enough long-distance colonization events to significantly impact plant dynamics on large scales. □

Methods

Setting

The study site is an 80–100-year-old oak–hickory forest composed of mixed hardwood species with *Quercus alba*, *Q. michauxii*, *Q. velutina*, *Carya tomentosa*, *C. ovata*, *Liriodendron tulipifera*, and *Liquidambar styraciflua* as canopy dominant (and *Pinus taeda* as a minor component), and mostly *Ostrya virginiana*, *Carpinus caroliniana* and *Cornus florida* in the understorey. The oldest individuals exceed 180 years. Tree density is 311 ha^{-1} , basal area is $26.3 \text{ m}^2 \text{ ha}^{-1}$, maximum leaf area index (projected foliage area per ground area) is 5.6, and maximum tree height is 33 m.

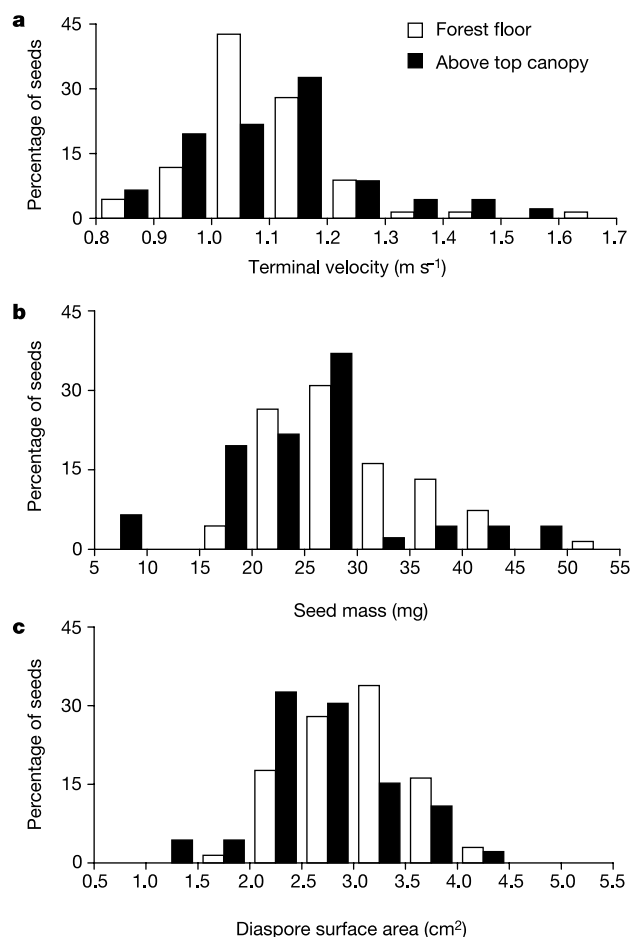


Figure 3 Frequency histograms of morphological traits of seeds collected at traps above the forest canopy top versus seeds collected on the forest floor during the autumn of 2001. There is no difference in terminal velocity (**a**) (mean $\pm$ s.e.: $1.12 \pm 0.02 \text{ m s}^{-1}$, $n = 46$ and $1.10 \pm 0.02 \text{ m s}^{-1}$, $n = 68$, respectively; t -test, $P > 0.5$), but seeds collected above canopy top are significantly lighter (**b**) (25.4 ± 1.3 and $29.5 \pm 0.8 \text{ mg}$, respectively; t -test, $P = 0.004$), and have smaller surface area (**c**) (2.20 ± 0.09 and $2.53 \pm 0.07 \text{ cm}^2$, respectively; t -test, $P = 0.003$). The Wald–Wolfowitz runs test reveals that for each of the three traits the observations of the two samples are randomly scattered throughout the ranking ($P > 0.1$). Therefore, despite the differences among means, the two samples appear to come from the same population.

We placed 102 seed traps at 12 levels along a 45-m high tower, with the three upper levels above the tallest trees (33 m). Traps were checked at weekly or biweekly intervals.

We surveyed all trees in a radius of 50 m around the tower. Trees were identified to species, mapped on a 2.5-m grid, and measured for DBH (diameter at breast height, 1.3 m). For a sample of at least 15 trees of each species, we measured tree height and regressed its logarithm against basal area. All regression slopes were significantly greater than zero ($P < 0.001$ in all cases) and basal area explained 61–85% of variance in tree height. We estimated height from the basal area using the regression function for trees whose heights were not measured.

We sampled the time series of the velocity components at 10 Hz using three triaxial sonic anemometers positioned at 40, 33 and 18 m above the forest floor. Plant area density (PAD) was measured with a LAI-2000 canopy analyser every 2 m, and leaf area density was inferred from PAD. A drag coefficient of 0.15 was chosen to match measured mean windspeed inside the canopy at these three levels.

Model simulations

We apply a coupled eulerian–lagrangian approach^{22,23} to simulate seed dispersal from trees around the tower. We calculated statistics of wind velocities (vertical, longitudinal and lateral) inside the canopy using an eulerian second-order closure model forced by the 30-min measured friction velocity (u^*) at 40 m. Closure models, and the mixing layer analogy that describes key length and timescales of organized eddy motion for canopy flows, have been reviewed by Finnigan²⁹. The lagrangian velocity used to model seed trajectories is constructed at 10 Hz in a manner that: (1) conserves coherency of intermittent eddies and (2) when averaged at a given canopy layer recovers 30-min statistics computed by the eulerian model.

We ran spatially explicit simulations of seed dispersal from all reproductive adults in a radius of 50 m around the tower. Because *P. taeda* was rare near the tower, we extended the mapping radius to 150 m for this species. Based on local observations, we define adult trees as those having DBH ≥ 15 cm for all species but *C. caroliniana*, for which we set a threshold of 7 cm.

The number of seeds released from each tree was linear with basal area¹⁸. For each simulated dispersal event, release height and seed terminal velocity were randomly selected from a normal distribution (Table 1). For each species, we estimated the vertical distribution of seed release by counting seeds or inflorescences along tree height for at least five trees. On the basis of these observations, we calculated the mean release height (Table 1) and assumed a normal distribution with a standard deviation of 0.2 of local tree height. The mean and variance of terminal velocity were estimated from video photos of falling seeds (collected at the study site), for at least 100 seeds per species. We incorporated temporal variation in wind conditions by running the model for all 1,271 half-hour averages of u^* and wind direction recorded by the upper anemometer during the simulated period.

In Fig. 2 we examine whether the bimodal patterns observed in the dispersal kernels, given spatially constant wind conditions, remain persistent when spatial variation in winds is introduced. In the first set of simulations, we assume that u^* above the canopy is constant in the plane parallel to the forest floor. Its value is related to the time-averaged shear stress above the canopy ($\tau_t = \rho u^{*2}$, where ρ is the air density), typically produced by meso-scale pressure gradients. In the second set, τ_t varies randomly in space, while keeping the same mean as in the first set (and hence the same u^*). Because the mean horizontal windspeed and vertical velocity standard deviation at any height within (or above) the canopy scale linearly with u^* , probability density functions generated from uncorrelated random normal variations of u^* in space or time converge to the ensemble average by the ergodic theorem³⁰. For simplicity, the u^* variations in Fig. 2 were produced in time with a mean identical to the constant u^* scenario. However, they can be interpreted as variations of u^* in space when the number of seeds released is large (as is the case here).

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A neural correlate of response bias in monkey caudate nucleus

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Primates are equipped with neural circuits in the prefrontal cortex^{1–6}, the parietal cortex⁷ and the basal ganglia^{6,8–11} that predict the availability of reward during the performance of behavioural tasks. It is not known, however, how reward value is incorporated in the control of action. Here we identify neurons in the monkey caudate nucleus that create a spatially selective response bias depending on the expected gain. In behavioural tasks, the monkey had to make a visually guided eye movement in every trial, but was rewarded for a correct response in only half of the trials. Reward availability was predictable on the basis of the spatial position of the visual target. We found that caudate neurons change their discharge rate systematically, even before the appearance of the visual target, and usually fire more when the contralateral position is associated with reward. Strong anticipatory activity of neurons with a contralateral preference is associated with decreased latency for eye movements in the contralateral direction. We conclude that this neuronal mechan-

ism creates an advance bias that favours a spatial response when it is associated with a high reward value.

Reward shapes goal-oriented behaviour^{12–14}. Consider a visually guided saccade task with an asymmetrical reward schedule (Fig. 1a and b, biased saccade task; BST). The monkey has to make an immediate saccade to a peripheral visual target in every trial, but is rewarded for a correct saccade to only one out of two possible target positions. The mapping between target position and reward remains constant for 20 or 40 trials, but is reversed frequently during an experimental session (Fig. 1c and Methods).

In this task, the average latency of saccadic eye movements was reliably shorter in trials with reward than in trials without reward. Figure 1d shows the rightward saccades of monkey 1; saccade latency was 88 ms shorter in reward than in no-reward trials ($P < 0.01$, two-tailed t -test). The reward effect amounted to 53 ms for the leftward saccades of monkey 1, 141 ms for the rightward saccades of monkey 2, and 203 ms for the leftward saccades of monkey 2; all three effects were significant ($P < 0.01$, two-tailed t -test). Although previous research has identified neural circuits that encode the presence of reward^{1–11}, little is known about how such representations may cause the reward effect in the behaviour. Here we explored the possibility that the brain creates a reward-oriented response bias before the presentation of an instruction on where to move the eyes.

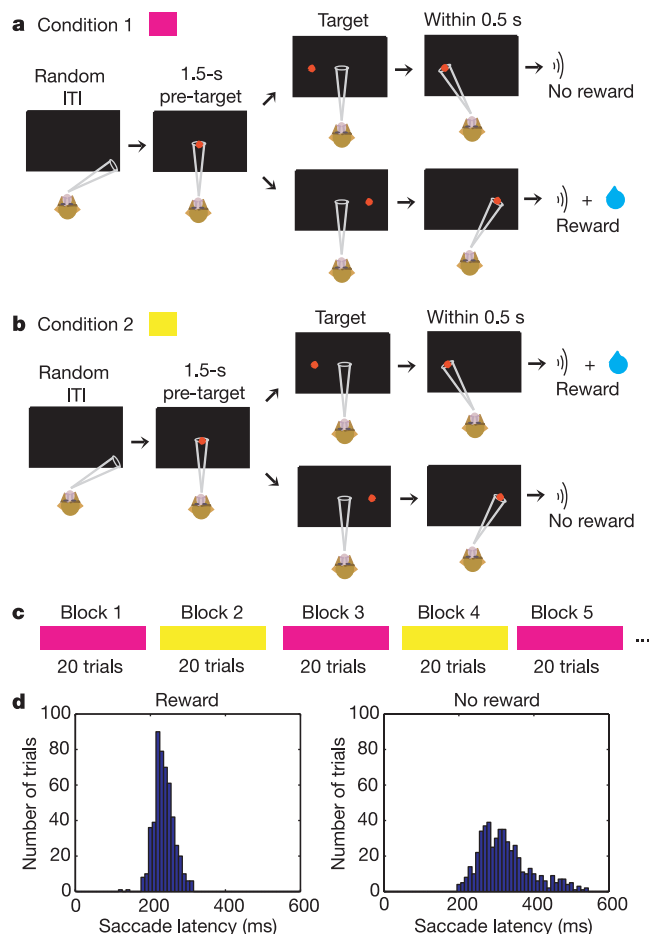


Figure 1 Experimental design of the biased saccade task (BST). **a**, Sequence of events in condition 1, in which only correct rightward saccades are rewarded. **b**, Sequence of events in condition 2, in which only correct leftward saccades are rewarded. **c**, Blocked ('ABA') design with frequent repetitions of different conditions. **d**, Density function of saccade latency in reward trials versus no-reward trials (data from monkey 1, rightward saccades).

We concentrated on caudate neurons that selectively increased their discharge rate right before the expected appearance of a task-relevant visual target^{8,15–16}. On the basis of their discharge characteristics¹⁷, we assumed that these neurons were medium-spiny¹⁸, GABA (γ -amino butyric acid)-mediated¹⁹ projection neurons. Using a memory-guided saccade task²⁰, we previously found that

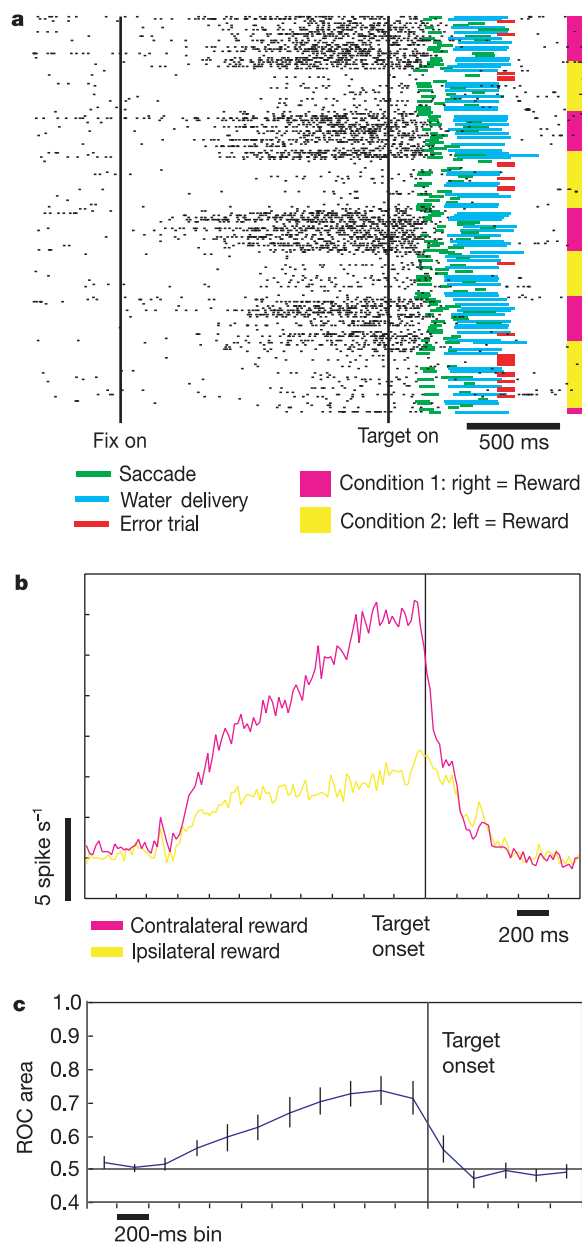


Figure 2 Effect of position-reward mapping on caudate anticipatory activity. **a**, Rasters of spikes aligned with target onset. Each raster represents one trial; trials are shown in order of presentation (from top to bottom). Green bars indicate saccade duration, blue bars indicate water delivery, red bars indicate error trial. Colour codes to the right of the rasters indicate the position-reward condition (yellow, left reward; purple, right reward). **b**, Population histograms of the activity of caudate neurons with a contralateral pre-target bias ($n = 25$). The histograms are aligned with target onset (yellow, ipsilateral reward; purple, contralateral reward). **c**, Area under the receiver operating characteristic (ROC area) in a sliding 200-ms window. Shown here is the average ROC area ($\pm 95\%$ confidence interval) from the same population of neurons as in **b**. The ROC area quantifies the separation of the distributions in the contralateral and ipsilateral reward conditions (0.5, no separation; 1, perfect contralateral preference; 0, perfect ipsilateral preference).

the anticipatory activity of these neurons is sensitive to the contingency between stimulus and reward^{21–22}. In the memory-guided saccade task, however, the delay between target presentation and saccade prevented us from studying the relationship between neuronal activity and behavioural parameters. Thus, to test whether caudate neurons create an advance response bias, we examined their activity in the BST, in which the monkey had to make an immediate visually guided saccade under an asymmetrical reward schedule. Forty-one caudate neurons with anticipatory activity provided sufficient data for analysis. Of these 41 neurons, 31 (76%) showed a reliable effect of position-reward mapping in their anticipatory activity ($P < 0.01$ in the window of -1.5 s to 0 s from target onset, two-tailed t -test), and usually fired more when the contralateral position was mapped onto reward than when the ipsilateral position was mapped onto reward (25/31; 81%). The remaining six neurons, with a preference for the ipsilateral-reward condition, behaved in all respects as the mirror image of neurons with a preference for the contralateral-reward condition.

The pre-target component in the caudate neurons showed marked selectivity. For the neuron shown in Fig. 2a, for example, the session started with a right (or contralateral) reward block, followed by an ipsilateral-reward block, then a contralateral-reward block again, and so on. Clearly, the neuron fired consistently more strongly in the contralateral-reward condition than in the ipsilateral-reward condition. This highly adaptive pre-target component cannot be explained by arousal, fixation-related activity or any other interpretation that does not take into account the context of the position-reward mapping. In Fig. 2b and c, we present population

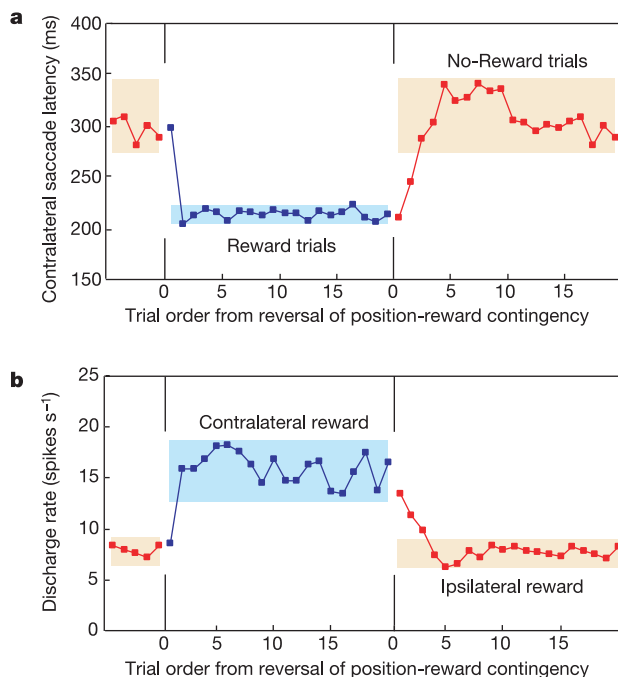


Figure 3 Adaptation of behaviour and neuronal activity to a reversal of the position-reward contingency. **a**, Latency for contralateral saccades as a function of trial order from reversal of position-reward contingency. Blue data indicate reward trials; red data indicate no-reward trials. Coloured backgrounds indicate the areas ± 2 s.d. from the mean in trials 6–20 (light blue, reward trials; light orange, no-reward trials). **b**, Neuronal discharge rate as a function of trial order from reversal of position-reward contingency. Shown are data from neurons with a contralateral pre-target bias (the same population of neurons as in Fig. 2b,c; same data set as in **a**). Blue data, trials in the contralateral-reward condition; red data, trials in the ipsilateral-reward condition. Coloured backgrounds indicate the areas ± 2 s.d. from the mean in trials 6–20 (light blue, contralateral-reward condition; light orange, ipsilateral-reward condition).

data of 25 caudate neurons that showed a similar preference for the contralateral-reward condition in their pre-target activity. The modulation by reward context started more than 1 s before target onset and reached a plateau by about 400 ms before target onset. The effect quickly dissipated after target onset. This time course clearly suggests that the pre-target component is prospective, in anticipation of the eye movement instruction.

For each of the 31 neurons with a reliable pre-target bias, we also obtained a reliable reward effect in the monkey's behaviour ($P < 0.01$, two-tailed t -test). This result confirmed that modulation of the pre-target component occurred together with the reward-dependent effects in saccade latency. Could the pre-target com-

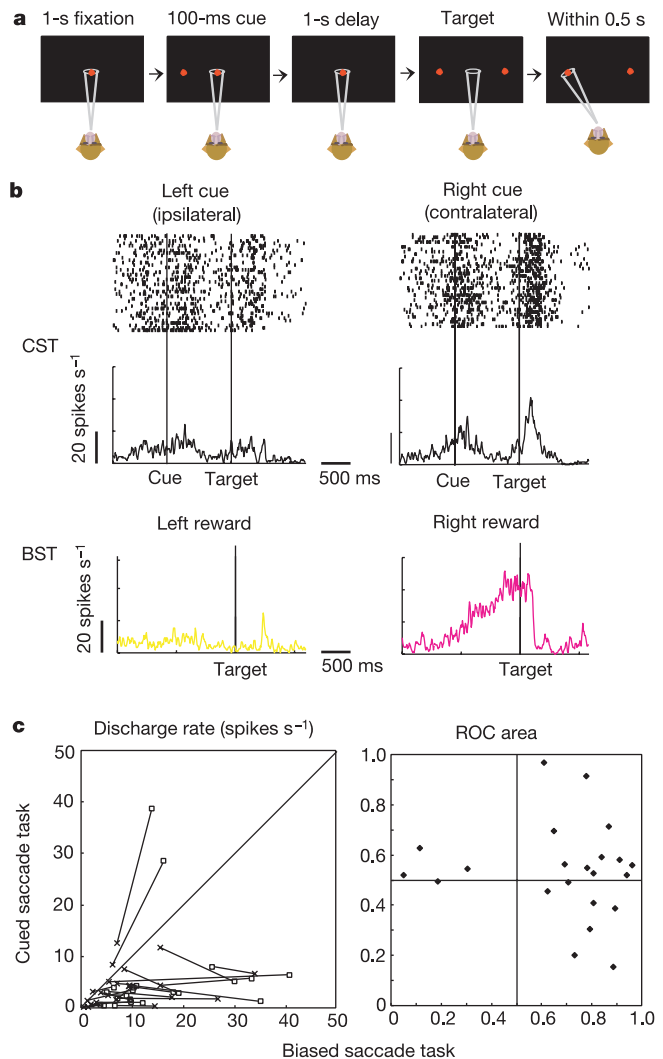


Figure 4 Comparison between the cued saccade task (CST) and the biased saccade task (BST). **a**, Sequence of events in CST. **b**, Top, rasters and histograms from a caudate neuron grouped by the spatial position of the cue in CST (top). Bottom, histograms for BST are shown for comparison (purple, left-reward condition; yellow, right-reward condition). **c**, Scatter plots of neuronal activity in CST versus BST. Data from 22 caudate neurons with a spatial pre-target bias in BST are shown. On the left, a line is drawn between two data points for each neuron: the data point indicated by a cross represents activity in the ipsilateral condition (vertical coordinate, ipsilateral cue in CST; horizontal coordinate, ipsilateral reward in BST); the data point indicated by a square represents activity in the contralateral condition in CST versus BST. On the right, each data point indicates the ROC areas of one neuron in the two tasks. ROC areas quantify the separation between ipsilateral and contralateral distributions (0.5, no separation; 1, perfect contralateral preference; 0, perfect ipsilateral preference). Vertical coordinate, ROC area in CST; horizontal coordinate, ROC area in BST.

ponent be a source of the reward effect in saccade latency? One interpretation is that the pre-target component acts as a spatial response bias, which prioritizes an eye movement toward the neuron's preferred position if that position is associated with a high reward value. Specifically, a strong pre-target component when the contralateral position is associated with reward will favour an eye movement to the contralateral position. When this position is no longer associated with reward, the weak pre-target component will lead to a reduced priority of an eye movement to the contralateral position.

The interpretation of caudate pre-target activity as a spatial response bias finds support in the way in which the monkey's behaviour and neuronal activity adapted to changes in the reward context (Fig. 3). We compared the latency for contralateral saccades with the pre-target activity (from -1.5 s to 0 s from target onset) of neurons with a preference for the contralateral-reward condition. Even though the monkey made saccades in each direction equally often (see Methods), we focused only on contralateral saccades for this analysis because we thought that there might be an association between contralateral saccades and the activity of neurons with a contralateral preference. Specifically, we examined how many trials it took for the contralateral-saccade latency and the selectivity in the neuronal activity to adapt to a reversal of the contingency between position and reward. In the latency for contralateral saccades (Fig. 3a), there was a notable asymmetry such that the reduction of latency in reward trials (the positive effect of incentive) was fully present from the second trial after a reversal, that is, immediately after the monkey experienced a change in reinforcement. The increase of latency in no-reward trials (the negative effect of no incentive) developed more slowly, reaching a peak at the fifth trial.

In the neuronal pre-target activity (Fig. 3b), we observed a similar asymmetry in the adaptation to a reversal. The increase of pre-target activity in the contralateral-reward condition was in full effect from the second trial after a reversal. This finding is consistent with the quick adaptation of the positive effect of incentive, assuming that strong pre-target activity leads to short latency for contralateral saccades. In contrast, the decrease of pre-target activity in the ipsilateral-reward condition developed more slowly, reaching a peak at the fifth trial. Again, this finding is consistent with the slow adaptation of the negative effect of no incentive, assuming that weak pre-target activity leads to long latency for contralateral saccades.

The slow versus fast adaptation after a reversal in the caudate pre-target activity might be related to different learning rates for positive-reward contingency (a contingency between the contralateral position and reward) than for negative-reward contingency (a contingency between the contralateral position and no reward)²³. Such asymmetry would in turn influence the development of the reward effect in the monkey's behaviour. Although the underlying mechanisms are not fully understood, the implication here is that there is tight coupling between the spatial pre-target bias in caudate neuronal activity and the reward effect in behaviour.

Although the pre-target component in caudate neurons seems to incorporate reward value in advance of the process of action control, it is possible that it represents an instance of a more general type of spatially selective anticipation. Specifically, previous research has documented spatially selective effects of target uncertainty on the anticipatory activity of neurons in parietal cortex⁷ and superior colliculus^{24,25}. Are effects of reward context and target uncertainty caused by similar neural mechanisms of anticipation? To test this possibility, we examined 22 of the 31 caudate neurons with a spatial pre-target bias in a second experiment, the cued saccade task (CST; Fig. 4). In this task, the monkey was required to make a saccade to a target that appeared at a previously cued position. The probability that a target appeared at the cued position was 100%. In half of the trials, we presented a distracter in addition

to the target, so that the monkey needed to process the cue position to distinguish target from distracter. Thus, correct task performance depended on the monkey's ability to select a spatial position on the basis of information provided in advance of target onset. Both monkeys performed this task with an error rate of less than 10% even in trials with a distracter, which indicates that they do rely on the spatial position of the cue.

Whereas the example neuron in Fig. 4b showed no effect of cue position in its activity in the window from -500 ms to 0 ms before target onset in the CST (Fig. 4b, top), it showed a clear contralateral pre-target bias in the same time window before target onset in the BST (Fig. 4b, bottom). For all but two neurons the laterality effect was stronger in the BST than in the CST (that is, longer extension in the horizontal than in the vertical axis; Fig. 4c, left), and there was no correlation between laterality effects in the different tasks (the Pearson correlation coefficient of the receiver operating characteristic (ROC) areas is -0.125 , which is not significantly different from zero; Fig. 4c, right). These results suggest that the caudate pre-target component is influenced by reward context, but not by target uncertainty. Further research is required to determine whether the caudate pre-target component can be influenced by other factors than reward.

In summary, the context of stimulus-reward mapping strongly modulates both the pre-target activity in caudate neurons and the latency of saccadic eye movements. The pre-target bias in caudate nucleus may be one of the ways in which the brain incorporates reward value in the control of action, even before the presentation of a visual instruction on where to move the eyes. By this interpretation, the bias in caudate nucleus favours a particular spatial response when it is associated with a high reward value, but not when it is associated with a low reward value. Such a response bias in the caudate nucleus could be propagated through a sequence of two inhibitory projections, from caudate nucleus onto substantia nigra pars reticulata, and then onto superior colliculus^{26–28}. Specifically, the caudate pre-target bias might lead to a spatially selective removal of tonic inhibition from substantia nigra pars reticulata on superior colliculus neurons with a particular movement field. The effect from caudate nucleus on superior colliculus would be to pre-establish a different baseline of neuronal activity in the contralateral-reward versus ipsilateral-reward condition. Thus, the caudate bias would bring superior colliculus neurons with a movement field to the position with a high reward value closer to initiating a saccade, even before the presentation of a visual instruction. □

Methods

Electrophysiological recording

We recorded from the caudate nuclei of two adult male Japanese monkeys (*Macaca fuscata*). Surgery was carried out under pentobarbital sodium anaesthesia to implant a head-holding device, a plastic unit-recording chamber and a scleral search coil. The recording chamber was placed over the fronto-parietal cortices, tilted laterally by 35° in the coronal plane, and aimed at the caudate head, as verified by magnetic resonance imaging (AIRIS, 0.3 T, Hitachi). Action potentials of single neurons were recorded with tungsten electrodes, which were advanced perpendicularly to the cortical surface using an oil-driven micro-manipulator (MO-95, Narishige). The action potentials were amplified, filtered (500 to 2,000 Hz) and processed by a window discriminator (MDA-4 and DDIS-1, BAK Electronics). We recorded eye movements using the magnetic search-coil technique (MEL-25, Enzanshi-Kogyo). All surgical and experimental protocols were approved by the Juntendo University Animal Care and Use Committee, and were in accordance with the NIH Guidelines for Care and Use of Animals.

Behavioural task

The monkey sat in a primate chair inside a sound-attenuated room with his head fixed. In the BST, the monkey was required to direct and maintain his gaze at a central fixation spot during a first fixation ('pre-target') period of 1.5 s. Visual stimuli were small red spots, 0.2° in diameter, back-projected onto a tangent screen by LED projectors. After the pre-target period, the fixation spot disappeared and a peripheral target appeared at 20° to the left or to the right. The monkey had to make a saccade within 500 ms to within 3° from the target position. A beep was presented as feedback for each correct trial. If the monkey made an error, the same trial was repeated. In half of the correct trials the monkey was rewarded with a drop of water depending on the reward schedule. During blocks of 20 or 40 correct trials, reward was mapped consistently onto one target position. We reversed the position-

reward contingency automatically (6–16 times) without any indication to the monkey.

There were various types of error trial: failing to fixate the fixation point, breaking fixation before fixation offset, making a saccade in the wrong direction, and making a hypo- or hypermetric saccade in the right direction. Because it can be difficult to relate caudate pre-target activity to the various types of error trial, we limited our focus in the present paper to correct trials. Our behavioural procedure ensured that we had an equal amount of data from correct trials in the reward condition and those in the no-reward condition.

In the CST, there was a first fixation period of 1 s, followed by a brief presentation (100 ms) of a cue at 20° to the left or to the right. The monkey had to maintain his gaze at the fixation spot during a delay period of 1 s. The fixation spot then disappeared and the target appeared at the same position as the cue. In half of the trials, another spot appeared at the alternative position (distracter). The monkey had to make a saccade to the target position within 500 ms, and was rewarded with a drop of water for a correct saccade. After recording neurons in the CST (experiment 2), we re-applied the BST (experiment 1) for at least 80 trials to confirm the reproducibility of neuronal activity.

Data analysis

Pre-target neurons were defined as neurons that showed a statistically reliable increase in the spike count –1.5 s to 0 s before target onset ('pre-target window') as compared with the spike count –3 s to –1.5 s before target onset. All pair-wise comparisons were evaluated by two-tailed *t*-tests, $P < 0.01$. We used the Bonferroni procedure to correct for family-wise error with multiple *t*-tests. To quantify the separation of population distributions from contralateral versus ipsilateral conditions, we calculated the area under the ROC in a sliding window of 200 ms.

To test the adaptation of saccade latency and pre-target activity to a reversal of position-reward contingency, we compared the second trial after a reversal against the third trial after a reversal (test 1). We also compared the second trial after a reversal against the pooled data from the sixth to twentieth trial (test 2). Both tests consisted of paired two-tailed *t*-tests on the mean data from individual neurons. Adaptation was judged complete if there was no significant difference between the measures. Tests 1 and 2 produced similar results in all cases.

For comparison between the two tasks (BST versus CST), we considered the neuronal activity from –500 to 0 ms before target onset in both tasks, in the computation of absolute firing rates as well as ROC areas.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Post-translational disruption of dystroglycan–ligand interactions in congenital muscular dystrophies

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Muscle–eye–brain disease (MEB) and Fukuyama congenital muscular dystrophy (FCMD) are congenital muscular dystrophies with associated, similar brain malformations^{1,2}. The FCMD gene, *fukutin*, shares some homology with *fringe*-like glycosyltransferases, and the MEB gene, *POMGnT1*, seems to be a new glycosyltransferase^{3,4}. Here we show, in both MEB and FCMD patients, that α -dystroglycan is expressed at the muscle membrane, but similar hypoglycosylation in the diseases directly abolishes binding activity of dystroglycan for the ligands laminin, neurexin and agrin. We show that this post-translational biochemical and functional disruption of α -dystroglycan is recapitulated in the muscle and central nervous system of mutant myodystrophy (*myd*) mice. We demonstrate that *myd* mice have abnormal neuronal migration in cerebral cortex, cerebellum and hippocampus, and show disruption of the basal lamina. In addition, *myd* mice reveal that dystroglycan targets proteins to functional sites in brain through its interactions with extracellular matrix proteins. These results suggest that at least three distinct mammalian genes function within a convergent post-translational processing pathway during the biosynthesis of dystroglycan, and that abnormal dystroglycan–ligand interactions underlie the pathogenic mechanism of muscular dystrophy with brain abnormalities.

Dystroglycan is the central protein in the dystrophin–glycoprotein complex (DGC), linking dystrophin in the intracellular cytoskeleton to proteins in the extracellular matrix^{5–7}. The biosynthesis of dystroglycan involves cleavage to generate the transmembrane β -dystroglycan (β -DG), which binds to the extracellular α -DG, both of which undergo extensive glycosylation^{8,9}. Despite the known involvement of DGC proteins in muscular dystrophies, mutations in dystroglycan have not been identified^{10–14}. In mice, disruption of the *DAG1* gene results in embryonic lethality, and normal dystroglycan expression seems to be essential for organization of basement membranes^{15,16}. We characterized dystroglycan protein localization in muscle biopsies of four MEB patients and three FCMD patients with a panel of dystroglycan antibodies. Both MEB and FCMD patients showed normal sarcolemma expression of β -DG (Fig. 1a). However, staining with monoclonal antibodies IIH6 and VIA4-1 failed to detect α -DG (Fig. 1a). To determine whether any portion of α -DG was expressed at the sarcolemma, biopsies were stained with a polyclonal α -DG antibody, GT20ADG (see Methods), which showed normal localization (Fig. 1a). These results indicate that α -DG is expressed at the membrane but that the epitopes for the monoclonal antibodies are specifically

disrupted in MEB and FCMD patients.

The epitope for IIH6 on α -DG can be disrupted *in vitro* by deglycosylation of α -DG⁷, suggesting that α -DG may be abnormally glycosylated in MEB and FCMD patients. Western blots of glycoproteins enriched with wheat germ agglutinin (WGA) agarose showed similar levels of β -DG expression, but no α -DG protein was detected with monoclonal antibodies IIH6 and VIA4-1 in MEB and FCMD patients (Fig. 1b, c). However, core α -DG polyclonal antibody showed that α -DG is expressed in MEB and FCMD patients at levels similar to control human muscle. Significantly, a reduction in relative molecular mass (M_r) of more than 50,000 (50K) in α -DG occurs in FCMD and MEB. More than half of the M_r of α -DG in normal muscle is glycoconjugates, and most of these glycoconjugates seem to be lost in both diseases. Interestingly, most α -DG migrates at similar M_r in FCMD and MEB, suggesting that the mutated genes associated with these two diseases might produce proteins that participate within the same enzymatic pathway, ultimately resulting in the transfer of sugars to α -DG. The rest of the components of the DGC, including dystrophin and the sarcoglycans, are normally expressed and glycosylated at the sarcolemmal membrane in MEB and FCMD (not shown).

Dystroglycan serves as a receptor for a variety of extracellular ligands, such as laminin⁸, agrin^{17,18} and neurexin¹⁹, in muscle, neuromuscular junctions, nerve and brain. Full chemical deglycosylation of α -DG *in vitro* can disrupt laminin binding activity^{7,19}. Therefore, we performed ligand overlay assays using laminin, neurexin and agrin on blots of WGA-enriched MEB and FCMD muscle (Fig. 2a, b). In muscle from MEB patients, ligand-binding activity of laminin, neurexin and agrin are each markedly diminished. Interestingly, in MEB patients the small amount of residual binding of ligands occurs in a dystroglycan species with higher M_r than most α -DG expressed. We suggest that a small amount of proper glycosylation occurs in the presence of mutated POMGnT1,

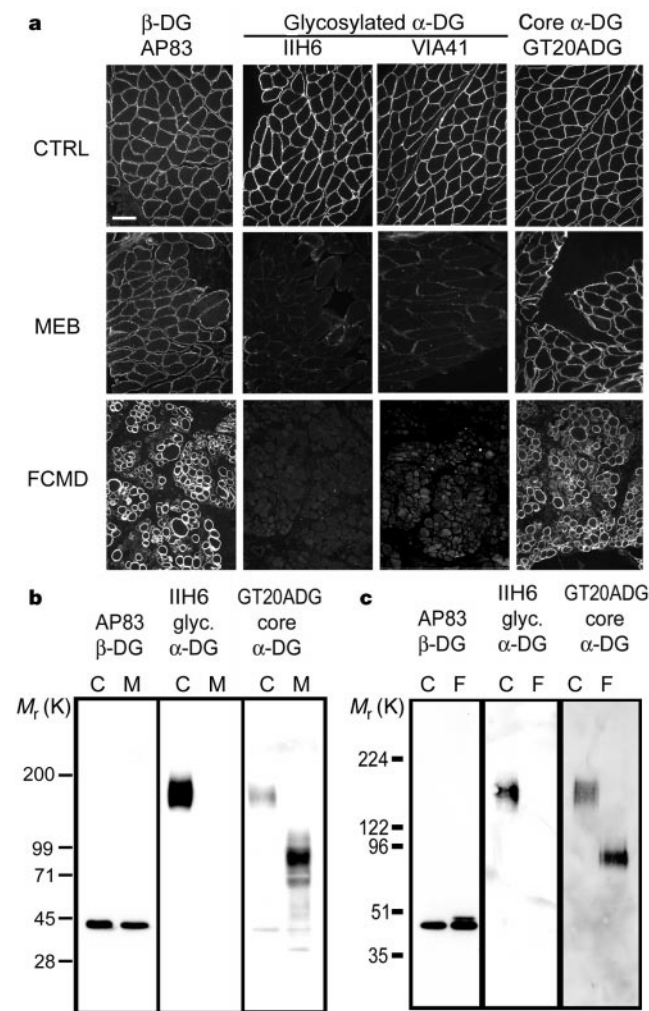


Figure 1 Post-translational modification of dystroglycan in MEB and FCMD.

a, Immunofluorescence localization of dystroglycan in biopsies of normal muscle (CTRL) and muscle of MEB and FCMD patients, using antibodies against β -DG, glycosylated α -DG, and α -DG core protein. Data shown are representative of all four MEB and all three FCMD patients. Scale bar, 100 μ m. **b**, **c**, Immunoblot analyses of age-matched normal (C) and MEB patient (M, **b**) and FCMD patient (F, **c**) WGA-enriched total muscle glycoproteins.

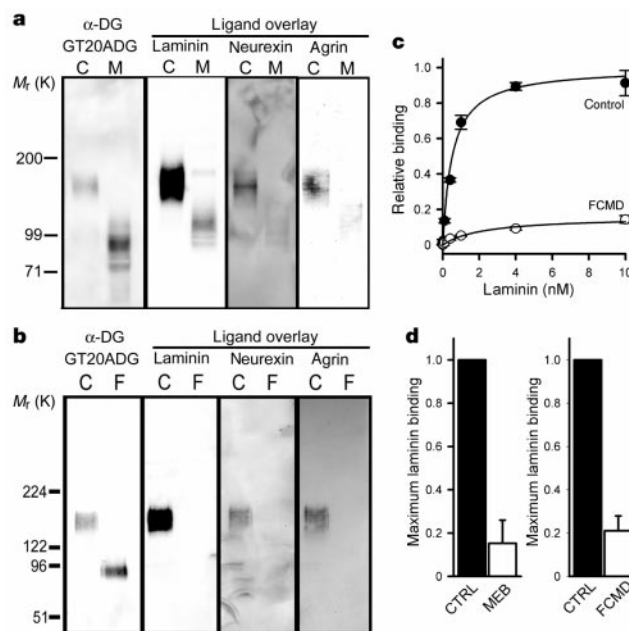


Figure 2 Dystroglycan–ligand interactions in MEB and FCMD. **a**, **b**, Ligand overlay assays on age-matched control (C) and MEB patient (M, **a**) and FCMD (F, **b**) WGA-enriched muscle homogenates using murine EHS laminin, neurexin fusion protein and recombinant rat agrin. **c**, Representative assay of solid-phase laminin-binding activity from control and FCMD muscle. Data are mean $\pm$ s.d. of triplicate samples fitted to a one-site model using the equation $A = B_{max}x/(K_d + x)$. **d**, Maximum laminin-binding activity in MEB ($n = 2$) and FCMD ($n = 3$) WGA glycoproteins relative to age-matched control muscle (CTRL). Data are mean $\pm$ s.e.m.

due either to residual enzymatic activity or to a secondary, partially compensatory enzyme. In FCMD patients, no binding of laminin, neurexin and agrin to α -DG is detected (Fig. 2b). To quantify the total ligand-binding activity of intact (non-denatured) α -DG, we performed solid-phase laminin-binding assays of WGA-enriched protein fractions from MEB, FCMD and age-matched control muscle (Fig. 2c, d). Nonspecific binding was calculated by the chelation of calcium with EDTA, which disrupts the laminin- α -DG interaction⁷. The quantitative solid-phase assays show a reduction of more than 80% in total high-affinity laminin-binding activity in MEB and FCMD muscle glycoproteins (Fig. 2d). Surprisingly, a small amount of residual binding activity remained in FCMD samples despite no activity by overlay assay (Fig. 2b, d). However, this residual activity in FCMD patients is insensitive to inhibition by the α -DG-blocking antibody IIH6, indicating that dystroglycan in FCMD is incapable of binding ligand (not shown). Total laminin-binding activity in age-matched control muscle was 80% inhibited by IIH6 (not shown). Because the residual high-affinity binding in FCMD is also insensitive to Arg-Gly-Asp (RGD) peptides (integrin inhibition, not shown), we suggest that this binding activity is due either to laminin in the preparation, or to an unknown, developmentally regulated laminin receptor in muscle. Thus, the lack of full glycosylation of α -DG in MEB and FCMD patients significantly disrupts the interactions of α -DG with extracellular matrix ligands known to be present in muscle, eye and brain.

The results in FCMD and MEB patients suggest that post-translational disruption of dystroglycan-ligand interactions may be a common mechanism for muscular dystrophy with brain abnormalities. A mutation in the *LARGE* gene, which encodes a

putative *N*-acetyl-glucosamine glycosyltransferase, has been linked to the muscular dystrophy phenotype in the *myd* mouse²⁰. In that study, muscle from *myd* mice showed a reduction in VIA4-1-reactive α -DG, but 'core antibodies' did not demonstrate any shift in the M_r of α -DG²⁰. Furthermore, no brain abnormalities have been reported in *myd* mice, suggesting that the effect of this putative enzyme to cause muscular dystrophy may be through a different mechanism or different receptor than in MEB and FCMD patients. We found that fully glycosylated α -DG was localized in muscle sarcolemma and astrocyte foot processes surrounding microvessels and abutting the glia limitans in the brain of wild-type mice (Fig. 3a). In addition, IIH6-reactive α -DG was found concentrated in puncta surrounding cerebellar Purkinje cell soma and throughout the molecular layer of cerebellum (Fig. 3a). In the *myd* mouse, IIH6-reactive α -DG in muscle and brain was not detected in any of these sites (Fig. 3a). IIH6-reactive α -DG is also expressed in the tissues of the eye: extra-ocular muscle membranes, retina inner limiting membranes, foot processes surrounding microvessels, and puncta in the outer plexiform layer (not shown). In the *myd* mouse, IIH6-reactive dystroglycan is absent from all of these sites, and although retinal neuronal layering appears grossly normal, extra-ocular muscle shows dystrophic features (not shown).

Western blotting of WGA-enriched homogenates from *myd* muscle and brain showed a biochemical phenotype remarkably similar to that of WGA-enriched MEB and FCMD patient muscle glycoproteins (Figs 1b, c and 3b). β -DG is normally expressed and glycosylated in *myd* muscle and brain, but α -DG reactive to IIH6 (Fig. 3b) and VIA4-1 (not shown) is absent. Core α -DG antibodies show that α -DG is expressed in *myd* muscle and brain but reveal a decrease in α -DG M_r similar to that of FCMD and MEB patients. The specificity of this glycosylation defect is indicated by normal expression of DGC proteins at the sarcolemma, normal sarcolemma lectin staining, and normal glycosylation of the sarcoglycans in *myd*

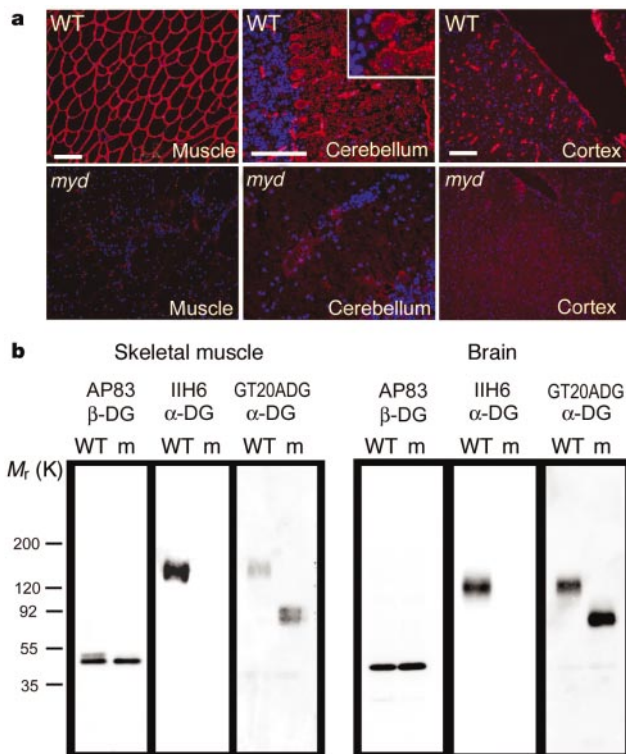


Figure 3 Post-translational modification of dystroglycan in *myd* muscle and brain. **a**, IIH6 immunolocalization in wild-type (WT) and *myd* muscle and brain (red). IIH6 epitopes on α -DG are lost in *myd* muscle sarcolemma and in astrocyte foot processes, and puncta in molecular layer (inset) in *myd* mice. Counterstain is 4,6-diamidino-2-phenylindole (DAPI), which labelled nuclei (blue). Scale bar, 100 μ m. **b**, Western blot analysis of dystroglycan expression in WGA-enriched homogenates of wild-type and *myd* (m) muscle and brain.

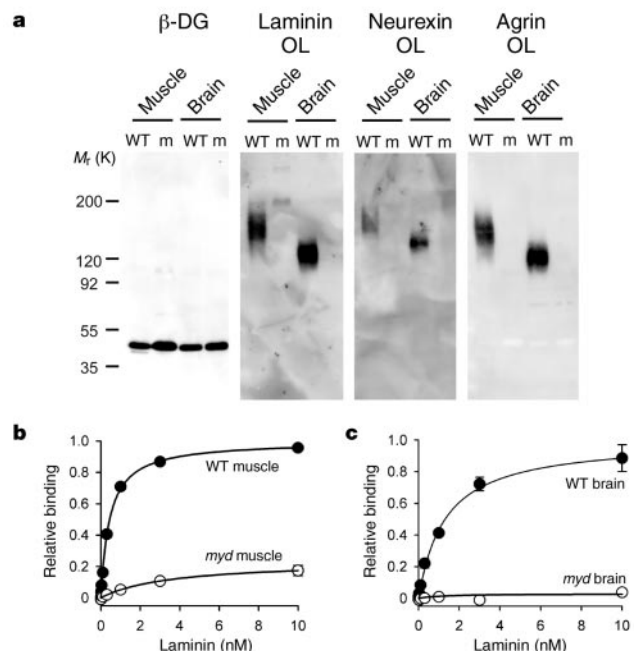


Figure 4 Dystroglycan-ligand interactions in *myd* muscle and brain. **a**, Ligand overlay (OL) assays on blots of WGA-enriched homogenates from *myd* skeletal muscle and brain. Lanes show control littermate mice (WT) and *myd* mice (m). **b**, **c**, Solid-phase laminin-binding assay in WT and *myd* WGA-enriched glycoproteins from skeletal muscle (**b**) and brain (**c**). A representative assay is shown with each binding condition performed in triplicate (mean $\pm$ s.d.).

skeletal muscle (not shown). Despite the differences in post-translational modification of α -DG in wild-type muscle and brain, the M_r of dystroglycan in *myd* muscle and brain is still slightly greater than the predicted M_r of the core protein ($\sim 75K$).

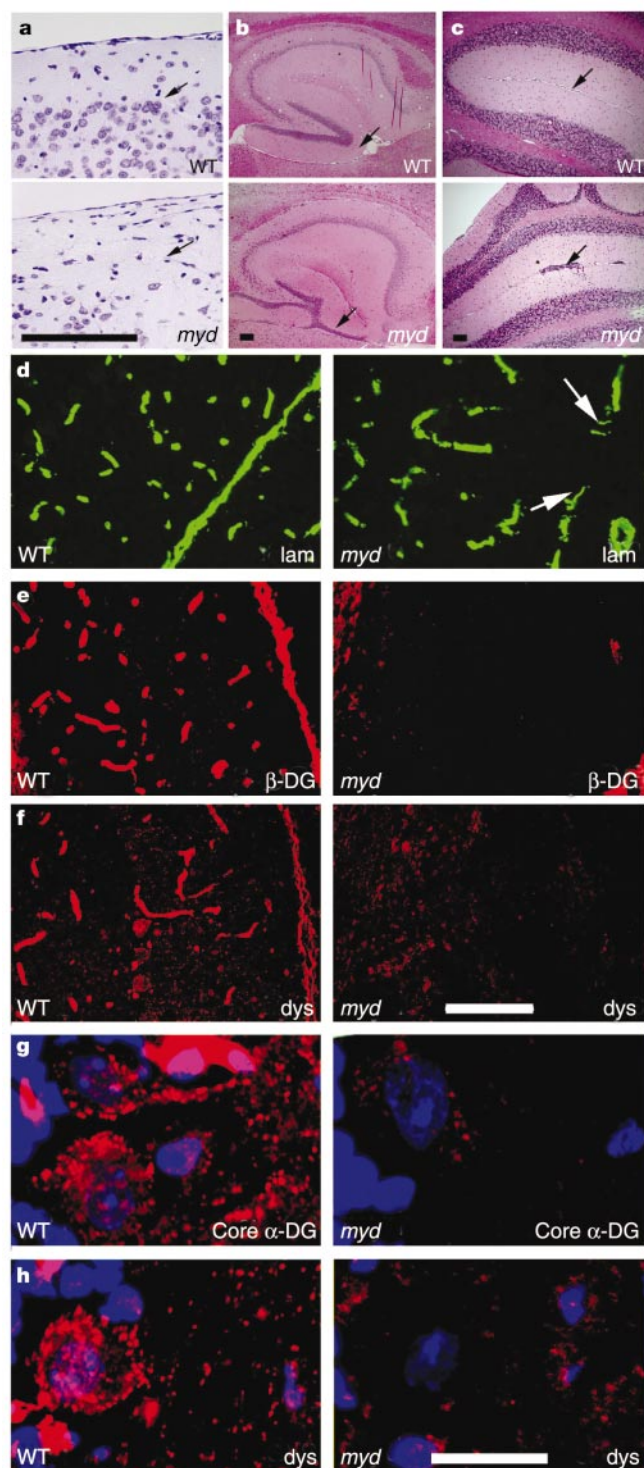


Figure 5 Abnormal neuronal migration and dystroglycan-associated protein targeting in *myd* mouse brain. **a**, Nissl-stained paired sagittal sections with boundaries between layer 1 and 2 in cerebral cortex (arrow). **b**, **c**, Abnormal migration of granule cells in *myd* dentate gyrus (**b**, arrow) and cerebellum (**c**, arrow). **d**, Polyclonal laminin (lam) antibody shows disruptions of glia-limiting membranes of cerebellum (arrows). **e**, **f**, Dystroglycan (**e**) and dystrophin (dys, **f**) targeting to cerebellar microvessels. **g**, **h**, Puncta staining with core α -DG (**g**) and dystrophin (**h**) antibodies in cerebellum (red). Counterstain is DAPI (blue). Scale bars: **a**–**f**, 100 μ m; **g**, **h**, 20 μ m.

Given that there were no previous reports of *myd* mice having brain abnormalities, we hypothesized that α -DG–ligand binding might be affected differently in *myd* muscle and brain than in MEB and FCMD muscle. However, ligand overlay assays showed that laminin, neurexin and agrin do not bind to α -DG from *myd* muscle and brain (Figs 3b and 4a). In addition, solid-phase binding assays of WGA-enriched glycoproteins show that high-affinity laminin binding is significantly reduced in *myd* muscle (Fig. 4b) and almost completely eliminated in *myd* brain (Fig. 4c). This suggests that the α -DG–ligand interactions are severely disrupted in *myd* mice, and that *myd* mice show a biochemical phenotype and M_r shift similar to MEB and FCMD patients.

Consistent with many of the brain abnormalities in MEB and FCMD patients, we show here that the *myd* mouse has a variety of neuronal migration abnormalities in cerebral cortex, cerebellum and hippocampus (Fig. 5). The cortical layering is disorganized, obscuring the well-defined boundary between layers I and II (Fig. 5a). The abnormal cortical layering is highly reminiscent of the type II lissencephalic changes seen in MEB and FCMD patients²¹. In cerebellum, populations of granule-cell neurons fail to migrate and are retained in clusters between adjacent folia (Fig. 5c). Granule-cell migration is also abnormal in hippocampus (Fig. 5b). The loss of α -DG–ligand interactions leads to several focal disruptions of glia-limiting basement membranes, noted by discontinuous laminin staining between adjacent folia in cerebellum (Fig. 5d). Furthermore, in contrast to *myd* muscle, which shows normal $\alpha 2$ laminin staining (not shown), $\alpha 2$ laminin staining is significantly reduced and pan-laminin antibodies show that laminin is mislocalized in cortex (not shown). In muscle, dystroglycan and DGC-associated proteins are expressed at the sarcolemma (see Supplementary Information). However, in brain the loss of the interactions between α -DG and the extracellular matrix leads to failure of α -DG, β -DG and dystrophin to concentrate to astrocyte foot processes (Fig. 5e, f, and Supplementary Information) and cerebellar puncta (Fig. 5g, h), although the expression of α -DG and β -DG appears similar in *myd* brain by western blot (Fig. 3b). Furthermore, proteins associated with DGC through dystrophin, including syntrophin and aquaporin-4, fail to target to the astrocyte foot process (see Supplementary Information). These results indicate that the loss of α -DG–ligand interactions disrupts the normal extracellular matrix composition and structure in important anchoring sites for supportive glial cells. Of equal importance, dystroglycan, through its interactions with extracellular ligands, seems to target proteins associated with the complex to the astrocyte foot process and the synapse.

The convergence of the post-translational perturbation and functional loss of α -DG–ligand interactions, muscular dystrophy and brain abnormalities in MEB, FCMD and *myd* mice provides strong evidence that α -DG is an important target protein that leads to disease. Importantly, the abnormal neuronal migration phenotype in *myd* mice is nearly identical to mice with a brain-specific dystroglycan gene deletion, demonstrating that the loss of dystroglycan–ligand binding activity is sufficient to account for this lissencephalic phenotype²². Indeed, the loss of dystroglycan–ligand binding activity in *myd* mice leads to a complete failure of targeting of dystroglycan and DGC proteins to important structural and function sites in brain, imparting a biochemical and functional dystroglycan deletion. These data provide strong support for the hypothesis that the tight link between the intracellular cytoskeleton and the extracellular environment through dystroglycan is central to the pathogenic mechanism of muscular dystrophy with abnormal neuronal migration.

The identification of an α -DG glycosylation defect in human MEB and FCMD patients highlights the importance of considering post-translational protein processing in genetic and acquired forms of human disease. Although fukutin belongs to the *fringe*-like family of glycosyltransferases, and LARGE has homology to an *N*-acetyl-

glucosamine transferase, neither has been demonstrated to have peptide transferase activity. Our results clearly show that a candidate glycoprotein substrate for these proteins shows a similar M_r shift with mutations in any of three distinct mammalian genes. This suggests that fukutin, LARGE and POMGnT1 may participate in a similar pathway that ultimately results in transfer of sugars to dystroglycan. Because *POMGnT1* is the only gene known to use O-linked mannosyl peptides as a substrate⁴ (a sugar peptide linkage unique to α -DG²³), we suggest that fukutin and LARGE may participate in either priming sugar donors for this reaction or cooperating with POMGnT1 in a larger macromolecular enzymatic complex. Finally, lymphocytic choriomeningitis virus (LCMV), which uses α -DG as a receptor, was recently shown to competitively inhibit α -DG interactions with laminin *in vitro*²⁴. Transplacental fetal LCMV infection produces developmental abnormalities in the central nervous system remarkably similar to MEB and Walker-Warburg syndrome²⁵. Therefore, disruption of dystroglycan–ligand interactions may be an important common mechanism to examine in a variety of genetic and acquired forms of neurological disorders that affect neuronal migration or synaptic integrity. □

Methods

Patient biopsies

Two siblings were diagnosed with Finnish MEB²¹. Genetic analysis of known mutated exons in *POMGnT1* revealed homozygosity for an IVS17 + 1 G-to-A splice site mutation previously described in the Turkish population⁴. One was a 2.5-year-old female with clinically diagnosed MEB or Walker-Warburg syndrome, based on the finding of congenital muscular dystrophy with eye and brain involvement. DNA for detailed genetic analysis was not available. The other was a 3.5-year-old male of Northern European and Slavic descent, with increased serum creatinine kinase, glaucoma, optic nerve colobomata/hypoplasia, abnormal T2 white matter and atrophy of the cerebellum and pons; he had a clinical diagnosis of MEB. The patient was heterozygous for a novel frameshift insertion in the tenth codon of exon 18 of *POMGnT1*. A second mutation was not found in known mutated exons 16–20 of *POMGnT1*, although a heterozygous polymorphism in intron 20 was observed. For MEB patients, control muscle was obtained from surgical discards of 12-year-old and 14-year-old females. FCMD patient tissue was obtained from three patients (aged 4–7 months) homozygous for retrotransposon insertions in the *fukutin* gene³. For FCMD patients, control muscle was from a 2.5-month-old sudden infant death syndrome patient, from the University of Miami Brain and Tissue Bank for Developmental Disorders. All tissue was obtained and tested in agreement with the Human Subjects Institutional Review Board of the University of Iowa.

Mice

Myodystrophy mice (*myd/myd*) and control littermate mice (+/+ or +/*myd*), 3–5 months old, were obtained from Jackson Laboratories.

Dystroglycan antibodies

VIA4-1 and IIH6 monoclonal antibodies recognizing α -DG were obtained from the University of Iowa Hybridoma Facility⁶. GT20ADG is from polyclonal antisera raised against the entire DGC²⁶ and purified against a hypoglycosylated full-length α -DG–human Fc fusion protein transiently expressed in HEK-293 cells²⁴. AP83 is a rabbit polyclonal antibody raised against the carboxy-terminal 15 amino acids of β -DG¹⁵.

Immunofluorescence microscopy

The primary antibodies for the DGC and laminin were used for immunofluorescence localization on human biopsies as described^{27–29}, and viewed on a Zeiss fluorescent microscope with digital imaging. For mouse brains, 7- μ m cryosections were post-fixed in 2% paraformaldehyde; antibody blocking and labelling was performed in PBS with 0.5% Triton X-100 and 5% BSA. Histological staining (haematoxylin and eosin, Nissl) was performed on brains that were perfusion fixed and embedded in paraffin.

Glycoprotein enrichment and western blot analysis

One hundred milligrams of muscle biopsy were solubilized in 1 ml of Tris-buffered saline (TBS) containing 1% Triton X-100 and protease inhibitors. The solubilized fraction was incubated with 200 μ l of WGA–agarose beads (Vector Labs) for 16 h. Pellets formed from the beads and these were washed three times in 1 ml TBS containing 0.1% Triton X-100 and protease inhibitors. The beads were then either directly boiled for 2 min in SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer (western blotting, ligand overlay) or eluted with 1 ml TBS containing 0.1% Triton X-100, protease inhibitors and 300 mM N-acetyl-glucosamine (solid-phase binding assay). Proteins were separated by 3–15% SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membranes and probed with dystroglycan antibodies described above or monoclonal antibodies recognizing sarcoglycan isoforms (Novacastra), and developed with horseradish peroxidase (HRP)-enhanced chemiluminescence.

Ligand overlay assay

Ligand overlay assays were performed on PVDF membranes using mouse Engelbreth–Holm–Swarm (EHS) laminin, α -neurexin–immunoglobulin (Ig) fusion protein¹⁹ or recombinant rat agrin (Chemicon). Briefly, PVDF membranes were blocked in laminin-binding buffer (LBB: 10 mM triethanolamine, 140 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.6) containing 5% nonfat dry milk (laminin or neurexin) or 5% BSA (agrin) followed by incubation of each ligand overnight at 4 °C in LBB. Membranes were washed and incubated with anti-laminin (Sigma) followed by anti-rabbit IgG–HRP, protein A–HRP (neurexin fusion protein) or anti-agrin (Chemicon) followed by anti-mouse IgG–HRP. Blots were developed by enhanced chemiluminescence (laminin and neurexin) or 4-chloro-1-naphthol (agrin).

Solid-phase assay

WGA eluates were diluted 1:50 in TBS and coated on polystyrene ELISA microplates (Costar) for 16 h at 4 °C. Plates were washed in LBB and blocked for 2 h in 3% BSA in LBB. Mouse EHS laminin was diluted in LBB and applied for 2 h. Wells were washed with 3% BSA in LBB, incubated for 30 min with 1:10,000 anti-laminin (Sigma) followed by anti-rabbit HRP. Plates were developed with o-phenylenediamine dihydrochloride and H₂O₂, reactions were stopped with 2N H₂SO₄, and values were obtained on a microplate reader. The data were fit to the equation $A = B_{\max}x/(K_d + x)$, where K_d is the dissociation constant, A is absorbance, and $B_{\max}$ is maximal binding.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy

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Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB), and Walker-Warburg syndrome are congenital muscular dystrophies (CMDs) with associated developmental brain defects¹⁻⁴. Mutations reported in genes of FCMD² and MEB⁵ patients suggest that the genes may be involved in protein glycosylation. Dystroglycan is a highly glycosylated component of the muscle dystrophin-glycoprotein complex⁶ that is also expressed in brain, where its function is unknown⁷. Here we show that brain-selective deletion of dystroglycan in mice is sufficient to cause CMD-like brain malformations, including disarray of cerebral cortical layering, fusion of cerebral hemispheres and cerebellar folia, and aberrant migration of granule cells. Dystroglycan-null brain loses its high-affinity binding to the extracellular matrix protein laminin, and shows discontinuities in the pial surface basal lamina (glia limitans) that probably underlie the neuronal migration errors. Furthermore, mutant mice have severely blunted hippocampal long-term potentiation with electrophysiologic characterization indicating that dystroglycan might have a postsynaptic role in learning and memory. Our data strongly support the hypothesis that defects in dystroglycan are central to the pathogenesis of structural and functional brain abnormalities seen in CMD.

The dystrophin-glycoprotein complex (DGC) of striated muscle is a well-characterized array of cytoplasmic, membrane-spanning and extracellular proteins that form a critical linkage between the cytoskeleton and the basal lamina⁸. The central protein linking DGC to the basal lamina is dystroglycan, a high-affinity receptor for several proteins (for example, laminin, agrin, neurexin and perlecan) that is composed of α - and β - subunits encoded by a single gene^{6,9}. α -dystroglycan (α -DG) is a highly glycosylated extracellular component, whereas β -DG spans the plasma membrane forming a

bridge between α -DG and the cytoskeleton⁶. Within the central nervous system (CNS), dystroglycan is abundant at two basal lamina interfaces formed by astrocytes: foot processes abutting on cerebral blood vessels, and foot processes that constitute the glia limitans at the pial surface of the brain and spinal cord⁷. Brain dystroglycan has also been localized to neuronal elements in several locations, including hippocampus and cerebellar cortex, where it may form a structural element of certain synapses⁷.

To explore the function of dystroglycan in brain and to determine whether dystroglycan operates in the pathogenesis of brain abnormalities such as those seen in CMD, we used Cre-loxP methodology to selectively delete dystroglycan from the CNS. Brain-selective expression of Cre recombinase was accomplished using a human glial fibrillary acid protein (GFAP) promoter expressed as early as embryonic day 13.5 (ref. 10). Studies in reporter mice reveal expression in astrocytes, oligodendroglia, ependyma and a large proportion of neurons¹⁰. It is likely that the human GFAP promoter is transiently active in neural progenitor cells, resulting in Cre recombination of floxed genes (genes flanked by loxP elements) that persist for the lifetime of several CNS cell lineages.

We produced both GFAP-Cre/DG^{lox/-} and GFAP-Cre/DG^{lox/lox} (GFAP-Cre/DG-null) mice that at weaning fit the expected mendelian distribution of each genotype. GFAP-Cre/DG-null mice are fertile and have no gross neurological abnormalities. Dystroglycan is largely absent from CNS tissue of GFAP-Cre/DG-null mice (Fig. 1a

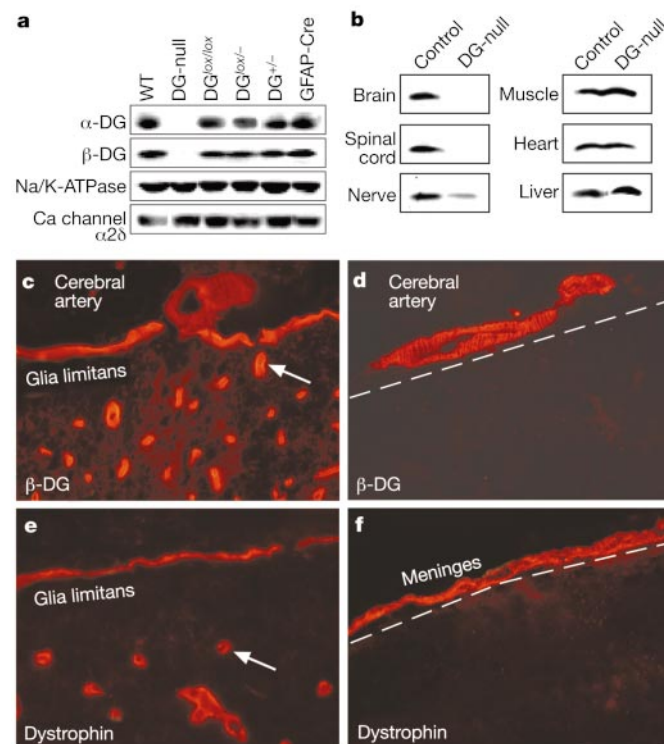


Figure 1 Tissue-selective deletion of dystroglycan. **a**, Western blot analysis of brain. WT, wild type; DG-null, GFAP-Cre/DG-null; DG^{+/+}, heterozygous dystroglycan null; DG^{lox/-}, heterozygous floxed/dystroglycan null; DG^{lox/lox}, homozygous floxed. **b**, Western blot with a β -DG antibody. **c**, β -DG immunofluorescence of control cerebral cortex shows staining of smooth muscle in a small cerebral artery on the pial surface and astrocyte foot processes along the glia limitans and cerebral microvessels (arrow points to one microvessel). **d**, β -DG immunofluorescence is retained in only a small cerebral artery on the surface of DG-null cerebrum. **e**, Dystrophin immunofluorescence parallels that of β -DG in control brain. **f**, Dystrophin immunofluorescence is retained only in vessels of the meninges of DG-null brain. The position of the unstained glia limitans is marked by a dashed white line in **d** and **f**.

and Supplementary Information). Tissue-selective deletion of dystroglycan was verified by polymerase chain reaction (PCR) and western blotting, showing dystroglycan gene recombination and protein ablation only in brain, spinal cord and peripheral nerve (Fig. 1b and Supplementary Information). Immunofluorescence evaluation parallels the western blots and demonstrates the loss of dystroglycan from astrocytic foot processes abutting the glia limitans and cerebral microvessels (Fig. 1d). In addition to dystroglycan loss, dystrophin isoforms are not expressed at these pial and perivascular locations in GFAP-Cre/DG-null mice (Fig. 1f). This suggests that the absence of dystroglycan has disrupted the DGC at these sites. Dystroglycan and DGC expression is, however, main-

tained in vascular smooth muscle of cerebral blood vessels (Fig. 1d, f) and in choroid plexus (data not shown).

The loss of dystroglycan leads to a number of structural developmental defects in every GFAP-Cre-expressing brain examined on both DG^{lox/lox} and DG^{lox/-} backgrounds. These abnormalities range from macrocephaly to a variety of neuronal migration errors. An increase in brain size of nearly 20% is evident from gross observations and from comparing brain mass from GFAP-Cre/DG-null mice and their sex- and age-matched littermates ($P \ll 0.05$; see Supplementary Information). Histopathologic abnormalities similar, but not identical, to CMDs in the DG-null mice include fusion of the cerebral interhemispheric fissure and adjacent cerebellar folia, multifocal disarray of neuronal layering in the cerebral cortex, malformations resembling polymicrogyria, and relatively well-defined heterotopia within the superficial cortex (Fig. 2). Minor dispersion of neuronal cell bodies in the CA1 region and focal irregularities of the dentate granule cell layer are seen in the hippocampus of some DG-null mice, although major white-matter tracts are unremarkable (data not shown). All mice have well-formed corpus callosa, anterior cerebral arteries and lateral ventricles (Fig. 2b), thus distinguishing the midline fusion in DG-null mice from the malformation holoprosencephaly. Although foliation progresses in a reasonably normal fashion in DG-null mice (see

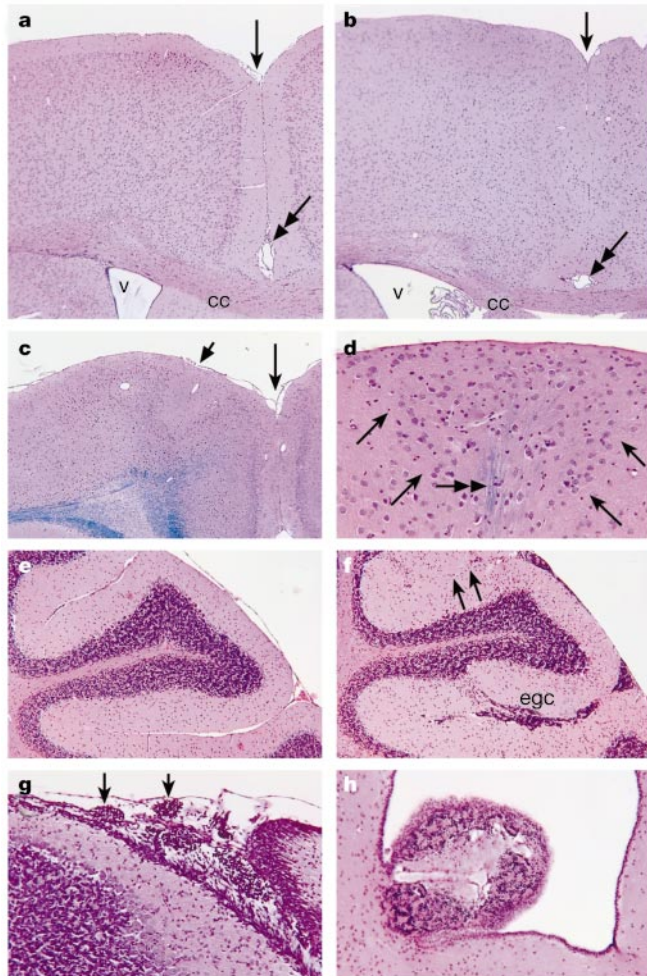


Figure 2 Abnormalities of cerebral and cerebellar development. **a**, Littermate control brain with normal interhemispheric fissure (vertical arrow), corpus callosum (cc), lateral ventricle (v) and anterior cerebral artery (double-headed arrow). **b**, GFAP-Cre/DG-null brain with fusion of the interhemispheric fissure, but normal corpus callosum, lateral ventricle and anterior cerebral artery. Abnormal neuronal migration has obscured the midline. Away from the midline there is disarray of cortical layering and migration of neurons into layer I, polymicrogyria (short arrow in **c**), and a distinct layer I heterotopia (outlined by single-headed arrows in **d**). A cluster of myelinated axons (double-headed arrow) passes through the cortex to this heterotopia. **e**, Para-sagittal section of control cerebellum. **f**, Para-sagittal section of GFAP-Cre/DG-null cerebellum shows abnormalities of external granule cell migration manifested by clusters of these neurons in one sulcus (egc) and along the pial surface (upper right area). There is fusion of another sulcus (arrows). **g**, Small clusters of external granule cells are abnormally placed in the subarachnoid space of a DG-null mouse at postnatal day 12 (arrows). **h**, Heterotopic cerebellar cortex forms a nodule protruding into the cerebral aqueduct of an adult DG-null mouse. **a, b, e-h**, Sections stained with haematoxylin and eosin. **c, d**, Sections stained with luxol fast blue.

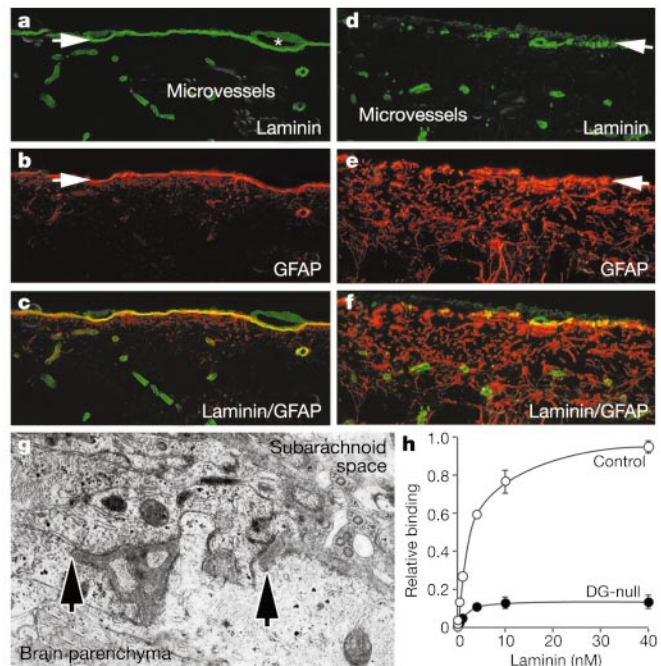


Figure 3 Disruptions of the glia limitans, leptomenigeal heterotopia and abnormal laminin binding. **a, d**, Laminin immunofluorescence (green) is present in the leptomeninges, pial vessels (asterisk in **a**), glia limitans (white arrows), and intracerebral microvessels of both control (**a**) and DG-null mice (**d**). The glia limitans is disrupted in DG-null mice (**d**). **b, e**, GFAP immunofluorescence (red) is prominent in only a superficial layer of astrocytes in control mice (**b**), but extends throughout the cortex in DG-null mice (**e**) (white arrows indicate glia limitans). **c, f**, Superimposing these images shows immunofluorescent overlap (yellow) between laminin and GFAP-stained astrocytic foot processes at the glia limitans and surrounding intracerebral blood vessels. Astrocytic processes extend through gaps in the glia limitans to form leptomenigeal heterotopia in DG-null mice (**f**). **g**, Electron microscopy confirms interruptions in the basal lamina of the glia limitans in DG-null mice (arrows), which should form a continuous horizontal band across the middle of this micrograph. Instead of being open, the subarachnoid space (upper portion of micrograph) is filled with cell processes. **h**, Total laminin binding is plotted for DG-null and littermate control brains. Binding in the absence of calcium was subtracted before plotting these curves.

Supplementary Information), there are residual nests of external granule cells in adult mice (Fig. 2f). Observations of granule cells in the subarachnoid space during postnatal cerebellar development in DG-null mice (Fig. 2g) suggest that abnormal migration and entrapment result in these granule cell nests. Additional heterotopic cerebellar tissue focally lines the subependymal regions of the fourth ventricle (see Supplementary Information) and forms nodules within the cerebral aqueduct (Fig. 2h).

Because dystroglycan is a major organizer of extracellular matrix proteins⁶, we hypothesized that the loss of dystroglycan from astrocyte foot processes at the glia limitans leads to basal lamina abnormalities that may underlie the neuronal migration errors present in our GFAP-Cre/DG-null mice. Indeed, immunofluorescence and ultrastructural analysis of both cerebral and cerebellar cortical surfaces of these mice reveals widespread discontinuities of the glia limitans accompanied by glial neuronal heterotopia within the leptomeninges (Fig. 3 and Supplementary Information; also see heterotopic granule cells in Fig. 2g). A profound reduction in the high-affinity laminin binding of DG-null brain (Fig. 3h) strongly suggests that the mechanism underlying the disruptions is a loss of ability to organize the basal lamina of the glia limitans. The basal lamina at this site is critical for normal cortical development. Enzymatic disruption or cold lesioning of this basal lamina causes abnormalities of cortical neuronal migration¹¹ and genetic disruption of perlecan or $\alpha 5$ -laminin, or combined disruption of $\alpha 3$ - and $\alpha 6$ -integrins, resulting in exencephaly, a severe defect in neural tube closure^{12–14}. Abnormalities of the glia limitans and meningeal heterotopia similar to our GFAP-Cre/DG-null mice have been reported after genetic deletion of $\beta 1$ -integrin, another membrane protein closely associated with the extracellular matrix¹⁵. Interruption of glia limitans integrity in GFAP-Cre/DG-null mice may alter positioning cues or disrupt the radial glia scaffolding necessary for normal neuroblast migration¹⁶. And we cannot rule out the possibility that a loss of brain dystroglycan may also alter adhesion of neuroblasts to radial glia.

The human condition most closely resembling the GFAP-Cre/DG-null mice is type II (cobblestone) lissencephaly¹⁶, the common brain abnormality seen in some types of CMD^{1–4}. That mutations in several genes of the DGC lead to muscular dystrophy in humans and

animals⁸ has led to speculation that a member of the DGC may be responsible for both the dystrophy and the CNS abnormalities in these CMDs. This hypothesis has become more credible since the cloning of *fukutin*, the gene responsible for FCMD², and the discovery of *POMGnT1* mutations in MEB⁵. Although the precise function of fukutin is not known, comparison of its amino-acid sequence to those of other known proteins suggests that fukutin may be a secreted glycosyltransferase¹⁷. *POMGnT1*, however, is a glycosyltransferase enzyme that catalyses *O*-mannosyl glycosylation⁵. Additional linkages between muscular dystrophy and glycosyltransferases have been shown in *myd* mice and humans with the discovery of mutations in *LARGE* and fukutin-related protein, respectively^{18,19}. This molecular data and the accompanying observations of abnormal α -DG expression^{5,18–20} have brought greater attention to dystroglycan as a candidate target protein in CMD, despite the lack of direct evidence that any of these proteins glycosylate dystroglycan. Our study shows that deletion of brain dystroglycan is sufficient to cause brain developmental abnormalities similar to the type II lissencephaly seen in FCMD, Walker-Warburg syndrome and MEB^{4,16}. Thus, brain-selective deletion of dystroglycan provides strong experimental support for the hypothesis that dystroglycan abnormalities underlie the CNS abnormalities in this group of CMDs.

In an accompanying study²¹, the potential role of dystroglycan glycosylation in the pathogenesis of CMD is supported by new data derived from MEB and FCMD patients and from *myd* mice. Skeletal muscle from MEB, FCMD and *myd* has abnormalities of α -DG glycosylation and profound reductions in high-affinity laminin binding. Reduced laminin binding in the brains of *myd* mice is accompanied by disruptions of the glia limitans and neuronal migration errors markedly similar to those of GFAP-Cre/DG-null mice. Therefore, loss of dystroglycan function through either genetic deletion or abnormal post-translational modification results in brain pathology like that seen in CMD.

We were surprised at the prominence of GFAP-immunoreactive astrocytes in the cerebral cortex of GFAP-Cre/DG-null mice (Fig. 3e). Although identifying a cause for this 'gliosis' will require further evaluation, disruption of the glia limitans between the brain parenchyma and the subarachnoid space may be responsible. The glia limitans at its interface between cerebral spinal fluid and the brain is similar to Reichert's membrane at its fetal-maternal interface in the developing embryo²². Both sites are proposed to provide a barrier and seem to have a high degree of dependence on dystroglycan for the development of a competent basal lamina. Loss of this glia limitans barrier, or perhaps abnormalities of the blood-brain barrier (another site from which astrocytic dystroglycan is lost), may lead to a reactive, inflammatory gliosis. Alternatively, altered neurogenesis or gliogenesis may underlie this striking astrocytic phenotype.

Dystroglycan is an important component of the neuromuscular junction, where it is proposed to organize postsynaptic components²³. Because dystroglycan is also localized to synaptic structures in sites such as the hippocampus⁷, we hypothesized that the broad deletion of dystroglycan from brains of these mice might alter synaptic function. Hippocampal slices prepared from 2–4-month-old GFAP-Cre/DG-null and littermate control mice were evaluated in a double-blind protocol that tested baseline neurotransmission, paired-pulse facilitation and long-term potentiation (LTP)—an electrophysiologic correlate of learning and memory²⁴. In control mice, robust LTP was consistently induced by high-frequency stimulation (HFS), whereas HFS induced only a short-term potentiation (STP) in the slices from DG-null mice (Fig. 4a). These results suggest that dystroglycan is required for HFS-induced LTP at CA3–CA1 synapses in the hippocampus.

In contrast to the blunted LTP, baseline neurotransmission was unaffected in the GFAP-Cre/DG-null mice (Fig. 4b). Stimulus-response relationships such as this are widely used to test the

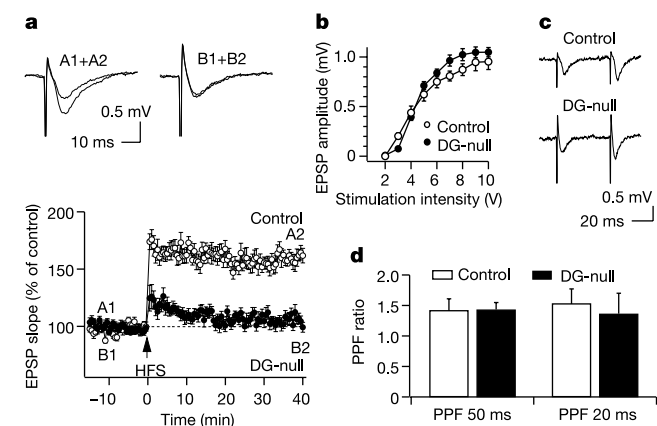


Figure 4 Long-term potentiation is blunted in GFAP-Cre/DG-null mice, but baseline neurotransmission and PPF are not affected. **a**, Representative EPSPs from control (A1, A2) and DG-null (B1, B2) mice (top). HFS induced LTP in control mice, but failed to induce LTP in DG-null mice. The slopes of the EPSPs before HFS served as control. The normalized slopes of EPSPs are plotted as a function of time. **b**, The stimulus-response curves from control and DG-null mice were produced by plotting the EPSP amplitudes against stimulation intensity. **c**, Representative raw PPF traces at 50-ms pulse intervals from control and DG-null mice. **d**, Averaged PPF ratios at 50- and 20-ms pulse intervals from control and DG-null mice.

properties of synaptic neurotransmission. Normal neurotransmission was observed despite the sporadic occurrence of the minor errors in hippocampal neuronal migration described above.

To determine whether the dystroglycan effect on LTP might be related to pre- or postsynaptic events, paired-pulse facilitation (PPF) was used to assess presynaptic neurotransmitter release²⁵. PPF with intervals of 50 and 20 ms did not differ significantly from each other in control and DG-null mice (Fig. 4c, d). These results indicate that the deficiency of dystroglycan does not affect presynaptic neurotransmitter release, and suggest that dystroglycan may contribute to the induction of LTP through an alternative, possibly postsynaptic, mechanism. This localization suggests a role for dystroglycan in CNS synapses similar to its role at the neuromuscular junction, where dystroglycan is proposed to organize the postsynaptic site^{6,23}. Alternatively, a growing literature indicating that astrocytes may integrate synaptic function²⁶ suggests that astrocyte abnormalities resulting from the loss of dystroglycan may account for the observed blunting of LTP in DG-null mice. □

Methods

Generation of floxed dystroglycan mice

A floxed DG targeting construct was generated with a floxed PGK-neo vector²⁷ that results in the insertion of a floxed *neo* cassette at the *SalI* site in the intron 5' of exon 2 and a *loxP* site at an *EcoRV* site 3' of exon 2, at a distance from the polyadenylation signal sequence (see Supplementary Information). This vector was electroporated into R1 embryonic stem cells and targeted clones were used to generate chimaeric animals. Germline transmission of the floxed DG allele was established by Southern blot and PCR screening of agouti offspring. Mice bearing the floxed DG allele were mated to the GFAP-Cre mice¹⁰.

Histology and immunohistochemistry

Histopathological studies were performed on mice of ages between postnatal day 12 and 52 weeks using standard haematoxylin and eosin, luxol fast blue, and Nissl staining methods. Tissues for electron microscopy were dissected from mice after transcardiac perfusion with 4% paraformaldehyde in PBS. Immunofluorescence was carried out on frozen sections using: polyclonal antibody against β -dystroglycan (rabbit 83)²⁸; monoclonal antibodies against dystrophin (MANDRA1) and GFAP (GFAP-Cy3) (Sigma); monoclonal antibody against α -dystroglycan (IIH6)²⁹; and polyclonal antibody against laminin (Sigma). Appropriately labelled secondary antibodies were obtained from Molecular Probes.

Biochemical analysis

Fresh tissue was disrupted sequentially by polytron and Dounce homogenization in 20 mM PBS, pH 7.1, 0.3 M sucrose, 1 mM MgCl₂, 0.5 mM EDTA, 0.6 mg ml⁻¹ pepstatin A, 0.5 mg ml⁻¹ leupeptin, 0.5 mg ml⁻¹ aprotinin, 0.75 mM benzamidin, 0.1 mM phenylmethyl sulphonyl fluoride, 0.4 mg ml⁻¹ calpain inhibitor 1, 0.4 mg ml⁻¹ calpeptin (Buffer A). The homogenate was centrifuged at 13,800g for 23 min at 4°C. The supernatant was centrifuged at 31,000g for 35 min at 4°C and the pellet was resuspended with Buffer A and subjected to SDS-polyacrylamide gel electrophoresis as a microsome fraction. Proteins were resolved by SDS-PAGE on 3–12% linear-gradient gels. Transfers were incubated with primary antibodies (α - and β -dystroglycan antibodies, as described above, Na/K-ATPase, and calcium channel, α 2^{28,29}) followed by appropriate peroxidase-conjugated secondary antibodies and developed using enhanced chemiluminescence (Super Signal, Pierce).

For laminin binding, 100 mg of mouse muscle or brain were solubilized in 1 ml of Tris-buffered saline (TBS) containing 1% Triton X-100 and protease inhibitors, and briefly centrifuged. Wheat germ agglutinin eluates were diluted 1:50 in TBS and coated on polystyrene enzyme-linked immunosorbent assay microplates (Costar) where binding assays with mouse Engelbreth-Holm-Swarm (EHS) laminin were carried out (see ref. 21).

Electrophysiology

Transverse hippocampal slices (350–400 μ m) from bregma -2.2 to -3.6 were prepared from control ($n = 8$) and GFAP-Cre/DG-null ($n = 9$) littermates at 2–4 months old, then studied without knowledge of the genotype. The brains were sectioned in ice-cold artificial cerebrospinal fluid at pH 7.4 containing (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.0 NaH₂PO₄, 1.3 MgSO₄, 26.2 NaHCO₃, 11 glucose, bubbled with 95% O₂ with 5% CO₂, and the slices incubated in the same solution at 31°C for 2–5 h before recording.

Standard extracellular field potential recordings were performed at 31 ± 0.5 °C³⁰. Field postsynaptic excitatory potentials (EPSPs) were evoked in CA1 stratum radiatum by stimulation of Schaffer collaterals with a bipolar electrode placed at the border of CA3–CA1 and recorded with a 3 M NaCl-filled glass pipette (<5 M Ω) using a biological amplifier (WPI, Iso-DAM8). Stimulation (100 μ s) was delivered every 30 s by a stimulus isolation unit (Grass, SD9). Stimulus-response curves were obtained by plotting the stimulus voltages against the amplitudes of the EPSPs. LTP was induced by a high-frequency stimulation (HFS; 100 Hz, 1 s) and was measured by normalizing the EPSP slopes after HFS to the mean slope of the baseline EPSP before HFS. Paired-pulse facilitation was observed by applying paired pulses with a 50- or 20-ms interval. Data were digitized (10 kHz), filtered at 1 kHz, monitored online, and stored for later analysis by

Pulse 8.41 (HEKA). The offline analysis was performed by PatchMachine (<http://www.hoshi.org>) and IgorPro version 4.0 (WaveMetrics). Unless otherwise noted, two-sample Student's *t*-tests were used to calculate the statistical significance.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Association of the *ADAM33* gene with asthma and bronchial hyperresponsiveness

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Asthma is a common respiratory disorder characterized by recurrent episodes of coughing, wheezing and breathlessness. Although environmental factors such as allergen exposure are risk factors in the development of asthma, both twin and family studies point to a strong genetic component^{1,2}. To date, linkage studies have identified more than a dozen genomic regions linked to asthma³. In this study, we performed a genome-wide scan on 460 Caucasian families and identified a locus on chromosome

20p13 that was linked to asthma (log₁₀ of the likelihood ratio (LOD), 2.94) and bronchial hyperresponsiveness (LOD, 3.93). A survey of 135 polymorphisms in 23 genes identified the *ADAM33* gene⁴ as being significantly associated with asthma using case-control, transmission disequilibrium and haplotype analyses ($P = 0.04$ – 0.000003). ADAM proteins are membrane-anchored metalloproteases with diverse functions, which include the shedding of cell-surface proteins such as cytokines and cytokine receptors⁵. The identification and characterization of *ADAM33*, a putative asthma susceptibility gene identified by positional cloning in an outbred population, should provide insights into the pathogenesis and natural history of this common disease.

A genome-wide scan for asthma was conducted on 460 Caucasian affected sib-pair (children having the same biological parents) families collected in the UK and the US. The initial linkage analysis was performed using a phenotypic definition of 'asthma' that required a physician's current diagnosis of asthma, as well as a requirement for active asthma medication use. Multipoint linkage analyses resulted in two peaks at 20p13; a maximum LOD score (MLS) of 2.94 near D20S906 and a second MLS of 2.94 at D20S482 (Fig. 1a). At both locations, the probability that two affected siblings shared two alleles identical by descent (IBD) was 31%, compared to the null expectation of 25%. To further characterize the linkage signal, more homogeneous phenotypes requiring both asthma and either bronchial hyperresponsiveness (BHR), elevated serum total IgE or positive specific IgE were analysed. Despite reducing the sample size to 218 nuclear families, the asthma plus BHR phenotype substantially increased the evidence for linkage (MLS of 3.93 at D20S482, 35% excess allele sharing) and refined the candidate region to the second peak (Fig. 1a). Although less dramatically than the asthma plus BHR phenotype, linkage analyses using asthma plus either elevated total IgE (274 families) or positive specific IgE (288 families) supported the region under the second peak with MLSs of 2.3 (32% excess allele sharing) and 1.87 (31% excess allele sharing), respectively (Fig. 1a). On the basis of the proportion of IBD, the phenotype of asthma plus BHR contributes most strongly to the linkage signal.

Gene discovery efforts were focused on a 1-LOD-drop support

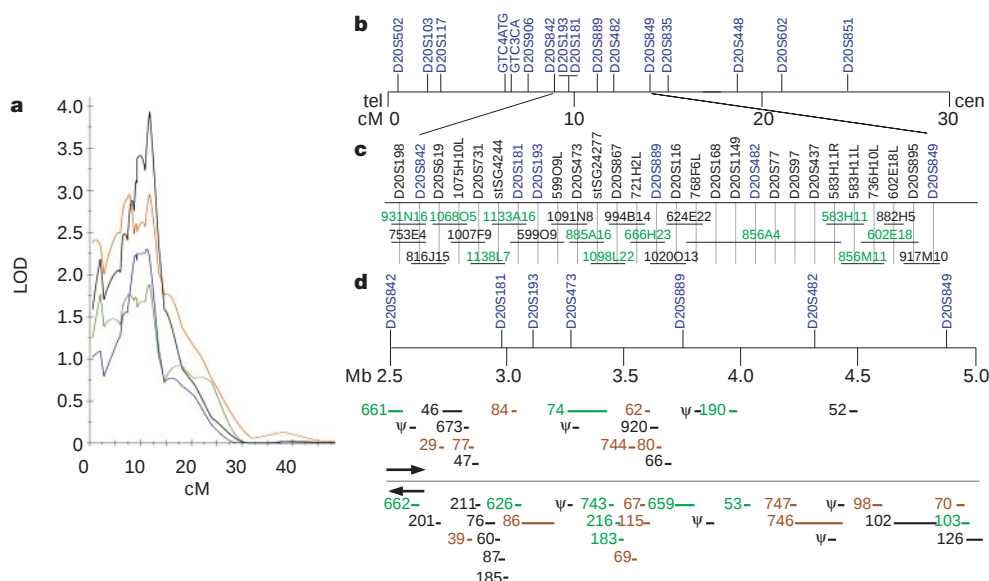


Figure 1 Linkage analysis and gene content of chromosome 20p13. **a**, lod score plots for four phenotypes: asthma (red), asthma and BHR (black), asthma and total IgE (blue) and asthma and specific IgE (green). Only the region of linkage is shown. **b**, Genetic map of the region of linkage (tel, telomere; cen, centromere). **c**, BAC contig. Genetic markers used in

genotyping (blue); BACs used in direct cDNA selection (horizontal lines); sequenced BACs (green). **d**, Gene content of the 90% confidence interval. Genes are indicated at the bottom with numbers (see Supplementary Information). Candidate asthma genes (green); other genes for which mutation analysis was performed (brown). Putative pseudogenes (Ψ).

interval around the MLS for the BHR phenotype, an approximate 90% confidence interval⁶. The region spanned 4.28 centimorgans (cM) (from 9.08 cM to 13.36 cM, Fig. 1b) within markers D20S842 and D20S849. A physical map was developed across this 2.5 megabase (Mb) region and selected bacterial artificial chromosome (BAC) clones were sequenced to facilitate gene identification (Fig. 1c). A combination of public data mining, complementary DNA (cDNA) library screening, direct cDNA selection and polymerase chain reaction with reverse transcription (RT-PCR) was used to characterize 40 genes (Fig. 1d, and Supplementary Information) that were prioritized for polymorphism identification and association studies based on their potential function, expression in relevant tissues and location with respect to the peak LOD score for BHR.

To determine whether polymorphisms in candidate genes were associated with asthma, association studies were performed using a case-control study design. Unrelated affected offspring from families showing evidence for linkage were selected as cases. Caucasian controls were collected in both the US and the UK in order to match the country of origin with the cases. On average, 130 IBD-affected individuals and 217 'hyper-normal' controls were compared for allele and genotype frequencies. A hierarchical strategy was adopted to detect association due to linkage disequilibrium between polymorphisms in the linkage interval and causative polymorphisms in candidate asthma genes. Analyses of 135 nucleotide polymorphisms (SNPs) in 23 genes spanning the 90% confidence interval revealed that the *ADAM33* region showed the most significant association signal in the linkage region. Linkage disequilibrium studies of polymorphisms in five genes flanking *ADAM33* showed that the linkage disequilibrium extended up to 130 kilobases (kb) to the left of *ADAM33* and up to 40 kb to the right. Perhaps surprisingly, higher levels of linkage disequilibrium between *ADAM33* and adjacent genes were observed in the US

population (Fig. 2a). Within this ~185-kb linkage disequilibrium interval, 24 SNPs in three genes were significant in either the combined or separate US/UK analyses (Fig. 2b). The majority of these SNPs (14) were in *ADAM33*. In the combined population, only *ADAM33* had multiple SNPs (six) that were significant ($P = 0.03$ – 0.006). In the UK, seven SNPs in *ADAM33* were significant ($P = 0.04$ – 0.004). In the US, six SNPs in *ADAM33* were significant ($P = 0.03$ – 0.003), with adjacent genes also containing multiple significant SNPs (two in *GFRA4* and six in *SN*). The

Table 1 Case-control analysis of *ADAM33*

Single SNPs significant at $P < 0.05$

		Allele frequencies		
SNP Name	Allele	Control	Case	<i>P</i> -value
Combined UK and US				
Q - 1	C	85.0%	91.2%	0.02
S1	G	89.5%	94.6%	0.02
ST + 4	A	51.5%	60.1%	0.03
ST + 7	G	78.1%	85.8%	0.02
V - 1	C	85.2%	92.4%	0.006
V4	C	76.7%	83.6%	0.03
UK				
F + 1	G	64.1%	74.2%	0.03
Q - 1	C	86.1%	92.3%	0.04
S1	G	89.4%	95.2%	0.03
S2	G	72.9%	84.0%	0.004
ST + 4	A	48.0%	59.2%	0.02
V - 1	C	86.4%	93.8%	0.01
V4	C	75.4%	83.7%	0.03
US				
I1	G	87.2%	70.4%	0.01
L - 1	A	8.0%	22.2%	0.01
M + 1	G	92.0%	77.8%	0.01
T1	C	7.8%	24.1%	0.003
T2	T	7.4%	20.4%	0.02
T + 1	C	92.0%	80.0%	0.03
SNP haplotypes (2-at-a-time) significant at <i>P</i> < 0.001				
Combined		UK		
SNP pair	<i>P</i> -value	SNP pair	<i>P</i> -value	
ST + 4/ <i>V</i> - 3	0.00004	S2/ST + 4	0.0003	
ST + 4/ <i>V</i> - 2	0.00004	S + 1/ST + 4	0.0004	
V1/ <i>V</i> 4	0.0004	ST + 4/ST + 5	0.0005	
V2/ <i>V</i> 4	0.0004	ST + 4/ <i>V</i> - 3	0.000003	
V4/ <i>V</i> 5	0.0003	ST + 4/ <i>V</i> - 2	0.000005	

Unrelated asthmatic offspring, from families showing evidence for linkage, were selected as cases. For the single SNP analysis, significance was evaluated by a Fisher exact test. For the haplotype analysis, P-values were computed for the overall test that compares the four possible haplotypes in the cases and in the controls by a permutation test.

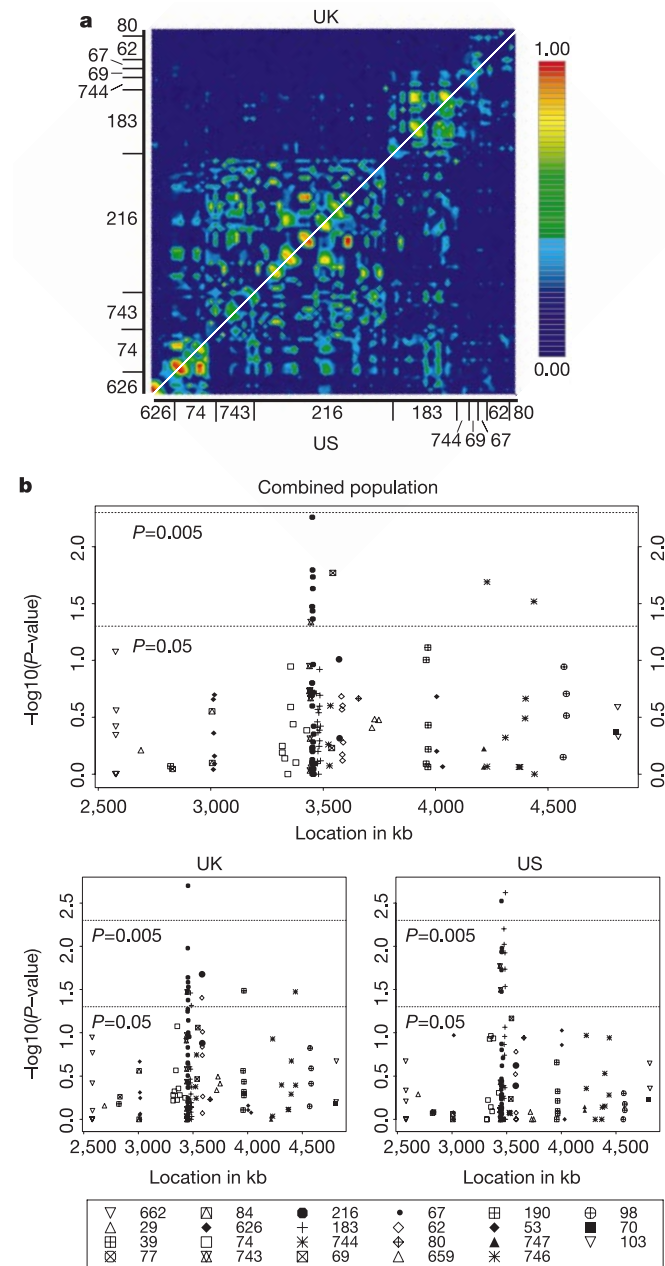


Figure 2 Linkage disequilibrium and case-control studies. **a**, Pairwise linkage disequilibrium between SNPs, as measured by Δ (colour scale) in the control populations, in the region of the *ADAM33* gene: upward triangle (UK population), downward triangle (US population). The graph is not to scale (SNPs are equidistant) to highlight the detailed pattern of linkage disequilibrium. Regions of high and low linkage disequilibrium are represented by red and blue shading, respectively. Boundaries and gene numbers are indicated along the horizontal and vertical axes. **b**, Case-control association plots ($-\log_{10}(P\text{-value})$) versus location in kb for the combined US and UK population (top), and the US and UK populations (bottom).

different pattern of linkage disequilibrium in the US and the UK samples was consistent with the pattern of association (additional significant SNPs in flanking genes in the US) and provided additional support for *ADAM33* as the origin of the signal (Fig. 2a). In general, the alleles in *ADAM33* that increased susceptibility to asthma were common, with allele frequencies ranging from 20% to 95% (in the Case column of Table 1).

Because multiple SNPs may act in combination to increase the risk of asthma, haplotype pairs were constructed and their frequencies compared between cases and controls. With the exception of *ADAM33*, few significant SNP combinations were observed. In the combined population, single pairs of SNPs in genes *GFRA4* and *SN* were significant at the $P = 0.005$ level. In contrast, for *ADAM33*, greater significance was most often observed for haplotypes versus individual SNPs, in many cases by several orders of magnitude. In the combined population, five pairs of SNPs in *ADAM33* were significantly associated with asthma at the $P < 0.001$ level (Table 1), with the most significant pair reaching a P value of 0.00004. An additional 23 pairs were significant at the $P \leq 0.01$ level. In the UK sample, five pairs of SNPs were significant at the $P < 0.001$ level (Table 1) with the most significant pair reaching a P value of 0.000003. An additional 34 pairs were significant at the $P \leq 0.01$ level. In the US sample, 21 haplotype pairs were significant at the $P \leq 0.01$ level.

In order to validate the case-control studies, which could generate false positive results due to population admixture, a family-based test of association, the transmission disequilibrium test (TDT), was conducted. Five candidate SNPs in *ADAM33* (V4, V1, V - 1, T1 and S1) that were significant in the case-control studies were typed on the full population (~2,000 individuals) to determine whether particular alleles were preferentially transmitted to affected individuals. One of the alleles from SNP S1 was significantly over-transmitted to affected offspring ($P < 0.04$) with two other SNP alleles reaching borderline significance ($P = 0.07$). However, when analyses were performed using up to five SNP combinations, most had haplotypes preferentially transmitted to affected offspring.

Eight haplotypes were significant at the $P < 0.005$ level, and six achieved significance at the $P < 0.05$ level. For all single SNPs or SNP haplotypes, the allele that was significantly over-transmitted was more frequent in the cases than in the controls (see Supplementary Information). As the asthma plus BHR phenotype resulted in a substantial increase in the linkage signal, TDT analyses were repeated using the BHR phenotype. The majority of SNP haplotypes became even more significant in this analysis ($P = 0.03-0.0004$, see Supplementary Information). For the majority of single SNPs or SNP haplotypes (16 out of 17), the allele that was significantly over-transmitted was more frequent in the cases than in the controls (see Supplementary Information). In summary, the *ADAM33* gene is significantly associated with asthma using case-control, TDT and haplotype analyses ($P = 0.04-0.000003$). A quantitative trait locus for airway hyperresponsiveness (*bhr1*) was mapped to a region on mouse chromosome 2 (74cM) that is syntenic to 20p13 (ref. 7), which is very close to the location for the mouse orthologue of *ADAM33* (73.9cM).

The ADAM gene family is a subgroup of the zinc-dependent metalloproteinase superfamily^{8,9}. ADAM proteins have a complex organization that includes eight domains: signal sequence, pro-domain, catalytic, disintegrin, cysteine-rich, epidermal-growth-factor (EGF)-like, transmembrane and cytoplasmic (Fig. 3). BLAST results indicated that *ADAM33* was most closely related to human *ADAM12*, *ADAM15*, *ADAM19* and *Xenopus ADAM13*, a branch of the ADAM family that possesses proteolytic activity¹⁰⁻¹². Northern blot analysis revealed two transcripts: one band at 5.0 kb and a second of lower intensity at 3.5 kb (Fig. 4a). RT-PCR, performed on RNA from five different primary cell types derived from lung tissue, indicated that *ADAM33* was expressed in lung fibroblasts and bronchial smooth muscle, but not in bronchial epithelial cells (Fig. 4c). Northern blot analysis of total cellular and cytoplasmic RNA revealed that the 5.0-kb transcript was not present in the cytoplasm of primary cells from bronchial smooth muscle (Fig. 4), suggesting that the larger message may contain unspliced intronic regions. The kinetics and efficiency of splicing in

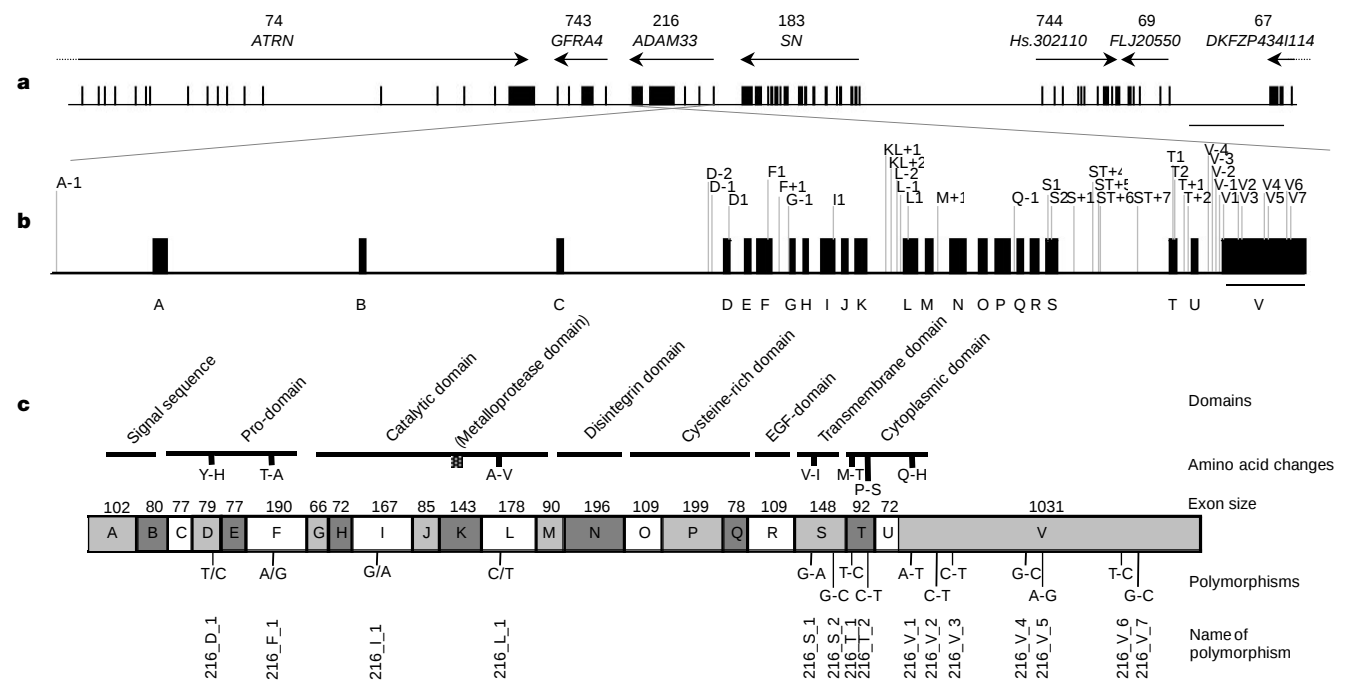


Figure 3 *ADAM33* chromosomal region. **a**, Genomic structure of genes flanking *ADAM33* (bar, 15 kb). **b**, The exon-intron structure of *ADAM33* (bar, 1 kb). SNPs in *ADAM33* are indicated above the gene. **c**, Domain organization of the *ADAM33* gene and location of coding and 3' UTR SNPs. Sizes of exons are given in base pairs (bp).

this gene may be controlled by regulatory elements located within introns of *ADAM33*¹³.

A total of 55 polymorphisms were identified in *ADAM33*, 37 of which were typed in the case-control studies (see Supplementary Information). Some of the significant SNPs were located in non-coding regions (within introns and the 3' untranslated region (UTR)) and may affect alternative splicing, splicing efficiency or messenger RNA turnover as reported for other disease-causing genes¹⁴. In the UK, significant SNPs and two-SNP haplotypes were primarily located in the 3' region of the gene and included an amino acid substitution in the transmembrane domain and an amino acid change in the cytoplasmic tail in combination with a SNP in the 3' UTR. In the US, a synonymous SNP in the catalytic domain was significant as well as two amino-acid changes in the cytoplasmic tail that may affect signalling (for example, SNP T2 is located within a putative SH3-binding domain).

Asthma is a chronic disorder in which T-cell-mediated inflammation causes thickening of airway walls, smooth muscle contraction, and narrowing of the airways. Epithelial damage, smooth muscle hyperplasia and increased matrix deposition are important characteristics of asthmatic airways that are thought to contribute to airway responsiveness¹⁵. This remodelling process has been linked to activation of the epithelial-mesenchymal trophic unit leading to proliferation of biosynthetically active fibroblasts, myofibroblasts and smooth muscle, a feature of BHR¹⁶. A role for *ADAM33* in airway remodelling and BHR is supported by the

linkage results reported above, as well as by expression in human lung fibroblasts and bronchial smooth muscle. The pattern of expression suggests that alterations in *ADAM33* activity or expression levels may underlie abnormalities in airway function rather than the immunological components of asthma. In addition, the known functions of ADAM family members in promoting myogenic fusion¹⁷ and the release of proliferative growth factors¹⁰ further support a role for *ADAM33* in airway remodelling. □

Methods

Family and clinical data collection

We collected 421 Caucasian affected sib-pair families, 323 from the UK and 98 from the US. An additional 39 families that met identical criteria were included from an earlier UK collection¹⁸. The case report form included questions on demographics, medical history including medication, a health survey on the incidence and frequency of asthma, wheezing, eczema, hay fever, nasal problems, smoking and home environment. Bronchial responsiveness was measured in subjects with a baseline forced expired volume in 1 s (FEV₁) of $\geq 70\%$ predicted using inhaled methacholine. Serum total and specific IgE levels (see below) were measured by the ImmunoCAPTM system (Pharmacia Diagnostics). The study received Institutional Review Board approval from participating collection sites and written informed consent was obtained from all human subjects.

Phenotype definitions

The criteria for being affected with asthma required a physician's diagnosis of asthma and current medication use. PC₂₀ (the provocation concentration of metacholine producing a 20% FEV₁ fall) was used to define a subset of asthmatic children with BHR (PC₂₀ ≤ 16 mg ml⁻¹). Asthmatic individuals were dichotomized using an age-specific cut-off for elevated total IgE levels (age 5–9 yr, >52 kilounits (kU) l⁻¹; age 10–14, >63 kU l⁻¹; age 15–18, >75 kU l⁻¹; age ≥ 19 , >81 kU l⁻¹). An individual was assigned a positive specific IgE value if his/her level was positive (to grass or tree) or elevated (≥ 0.35 kU l⁻¹ for cat, dog, *Dermatophagoides pteronyssinus*, *D. farinae*, *Alternaria*, ragweed) for at least one such measure. Caucasian controls that were negative for asthma were collected in the US (100) and the UK (200); a subset with negative skin prick tests (wheal ≤ 3 mm) for four common allergens (house dust mite, cat, grass and tree) were selected as 'hyper-normals'.

Genotyping

Genotypes of PCR-amplified simple sequence microsatellite genetic linkage markers were determined using ABI 377 Automated Sequencers (PE Applied Biosystems) using standard conditions. Microsatellite markers (Weber Set, version 8) were obtained from Research Genetics Inc. Markers GTC3CA (forward primer 5'-CTTCCTGCACTGAG GCTCTT-3', reverse primer 5'-ACAAGCCATGGGCTACAGAG-3') and GTC4ATG (forward primer 5'-CTCAGCCACTGTCTCTTCCC-3', reverse primer 5'-GTGTGAGAGTGTGAGCCAG-3') were derived from genomic sequence. Allele calling (binning) was performed using the SYBASE version of the ABAS software¹⁹. Binned alleles were imported into the program MENDEL²⁰ for inheritance checking and allele frequency estimation using the USERM13 subroutine²¹.

Linkage analysis

Multipoint linkage analyses of allele sharing in affected siblings were performed using MAPMAKER/SIBS²². The Kosambi mapping function was selected and sibships with s affected were weighted down to contribute an average of $(s - 1)$ affected sib-pairs to the likelihood (pair options, 3). The map location and distances between markers were obtained from published genetic maps (<http://www.marshmed.org/genetics/>). Ambiguous ordering of markers in the Marshfield map was resolved using the program MULTIMAP²³ applied to the 460 families in the study.

Gene characterization

BAC clones containing sequence tagged site markers were obtained by screening of the RPCI-11 human BAC library. Sequencing of BAC clones used standard ABI377 automated DNA sequencing methods. BAC DNA was pooled in equimolar amounts and used as the genomic template for direct cDNA selection as described²⁴. Directionally cloned cDNA libraries were constructed and screened by standard methods²⁵. RT-PCR was also used to isolate cDNA clones using a Superscript One Step RT-PCR kit (Invitrogen). Rapid amplification of cDNA ends was performed following the manufacturer's instructions using a Marathon cDNA Amplification Kit (Clontech). Northern blots were purchased from Clontech. Primary cell types were obtained from Clontech.

Association studies

SNPs were identified by screening 52 cases and 25 controls for polymorphisms using fluorescent single-stranded conformational polymorphism on ABI model 377 Automated Sequencers (PE Applied Biosystems) followed by sequencing of appropriate individuals. A combination of restriction fragment length polymorphism, allele specific oligonucleotide hybridization²⁶ and exonuclease proofreading methods (P. Cahill, manuscript in preparation) were used to type SNPs on the larger population. One affected sib was selected from each family containing an affected sib-pair sharing, with probability greater than 75%, either two alleles IBD or one allele IBD inherited from an affected parent. The frequency of the alleles and genotypes in the control and case populations were compared using a Fisher exact test. Linkage disequilibrium was measured in the US and UK control

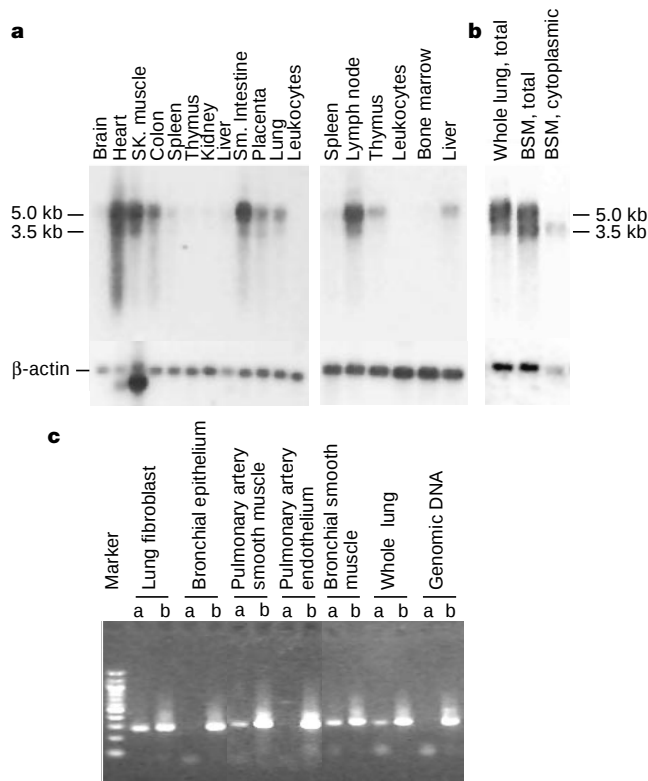


Figure 4 Expression analysis of the *ADAM33* gene. **a**, Northern blots hybridized with an *ADAM33* 3' UTR specific probe (top). Bottom, control human β -actin probe. **b**, Northern blots containing 1 μ g poly A⁺ RNA isolated from human whole lung, bronchial smooth muscle (BSM) cells (total RNA preparation) and BSM cells (cytoplasmic RNA preparation) were hybridized using the same 3' UTR specific probe. **c**, RT-PCR analysis of the expression of *ADAM33* in five different lung primary cell types and whole lung. Marker, 100-bp ladder. Lanes containing primers from *ADAM33* are labelled a, lanes containing primers from the ORF of β -actin are labelled b.

population with the absolute value of the correlation coefficient, Δ . Visual display of pairwise linkage disequilibrium among SNPs was produced with the program GOLD (<http://www.well.ox.ac.uk/asthma/GOLD/>)²⁷. Haplotype frequencies were estimated using an Expectation Maximization (EM) algorithm²⁸ based on a maximum likelihood approach; the algorithm was modified to allow for missing genotype information. Significance levels were computed using a permutation test. For a subset of the SNPs in the ADAM33 gene, a TDT²⁹ was conducted by using IBD transmission to both affected offspring. SNP haplotypes were constructed with GENEHUNTER³⁰ and used as 'alleles' in TDT analyses. Significance levels for the marginal homogeneity test were estimated by Markov chain Monte Carlo methods as implemented in a modified version of TDTEX (affected sib pair option) from the SAGE program.

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Supplementary Information is available on Nature's website (<http://www.nature.com/nature>).

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Competing interests statement

The authors declare that they have no competing financial interests.

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(e-mail: tkeith@genomecorp.com). Genbank accession numbers for the ADAM33 cDNAs are AF466287 and AF466289; the accession number for the genomic sequence is AF466288.

Short interfering RNA confers intracellular antiviral immunity in human cells

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Gene silencing mediated by double-stranded RNA (dsRNA) is a sequence-specific, highly conserved mechanism in eukaryotes. In plants, it serves as an antiviral defence mechanism^{1–3}. Animal cells also possess this machinery but its specific function is unclear^{4–10}. Here we demonstrate that dsRNA can effectively protect human cells against infection by a rapidly replicating and highly cytolytic RNA virus. Pre-treatment of human and mouse cells with double-stranded, short interfering RNAs (siRNAs) to the poliovirus genome markedly reduces the titre of virus progeny and promotes clearance of the virus from most of the infected cells. The antiviral effect is sequence-specific and is not attributable to either classical antisense mechanisms or to interferon and the interferon response effectors protein kinase R (PKR) and RNaseL. Protection is the result of direct targeting of the viral genome by siRNA, as sequence analysis of escape virus (resistant to siRNAs) reveals one nucleotide substitution in the middle of the targeted sequence. Thus, siRNAs elicit specific intracellular antiviral resistance that may provide a therapeutic strategy against human viruses.

Viral infection often results in the destruction of infected cells, which is mediated either by the virus itself or by the immune response; however, intracellular mechanisms that inhibit virus replication and enable viral clearance and cell survival seem to exist^{25,26}. The molecular basis for this virus clearance remains unclear. Recently, a dsRNA-mediated gene silencing (also called RNA interference, or RNAi) system has been observed in mammalian cells. This system uses 21-nucleotide dsRNA intermediates, siRNAs, to specifically downregulate cellular, as well as viral gene expression^{11–13}. These findings raise the question of whether gene silencing can be an effective mechanism of intracellular immunity, promoting viral clearance and cell survival. To determine whether RNAi can protect against a highly cytopathic virus, we studied the effects of siRNA on viral replication using poliovirus as a model. This positive-stranded RNA virus replicates in the cytoplasm of infected cells with rapid kinetics. Cells are lysed and viral progeny released within 6–8 h after infection. In addition, virally induced apoptosis is initiated soon after infection, resulting in rapid cell

death even in the absence of ongoing replication¹⁴. The effect of RNAi on viral replication was examined using two polio-specific siRNAs, one corresponding to a capsid sequence (siC) and another to a viral polymerase sequence (siP) (Fig. 1a). An unrelated siRNA corresponding to a firefly luciferase coding sequence (siL, Fig. 2b) was used to control for nonspecific dsRNA effects. Additional controls included the individual strands of siC (ssC(+)) and ssC(-)) and double-stranded DNA (C-DNA) of identical sequence to siC, which is unable to elicit RNAi⁶.

Strikingly, transfection of siC before infection with the virulent

poliovirus Mahoney strain completely inhibited virus plaque formation in HeLa cells. In contrast, none of the control transfections affected plaque formation (Fig. 1b). One-step growth curves indicated that cells treated with siC (Fig. 1c) or siP (not shown) produced only 1–3% of the virus titre observed in control-treated cells.

To examine the effect of siRNA on viral protein and RNA production, cells were infected at a high multiplicity of infection (MOI = 10). Although this treatment ensures infection of every cell, production of the viral polymerase (3D^{pol}) and its precursors was greatly impaired by treatment with siC and siP (Fig. 1d). Similarly, accumulation of poliovirus RNA in siC-treated cells was markedly reduced (Fig. 1e). Our results indicate that siRNA interferes with viral replication early after infection.

The effect of RNAi on viral replication was further investigated using poliovirus replicons, in which the capsid protein-coding sequences have been replaced by either firefly (FLuc) or renilla (RLuc) luciferase reporter genes (Fig. 2a). Cells were transfected with a 1-to-1 mixture of both replicons, plus siRNAs (Fig. 2c). Although siL had no effect on virus production (Fig. 1c), co-

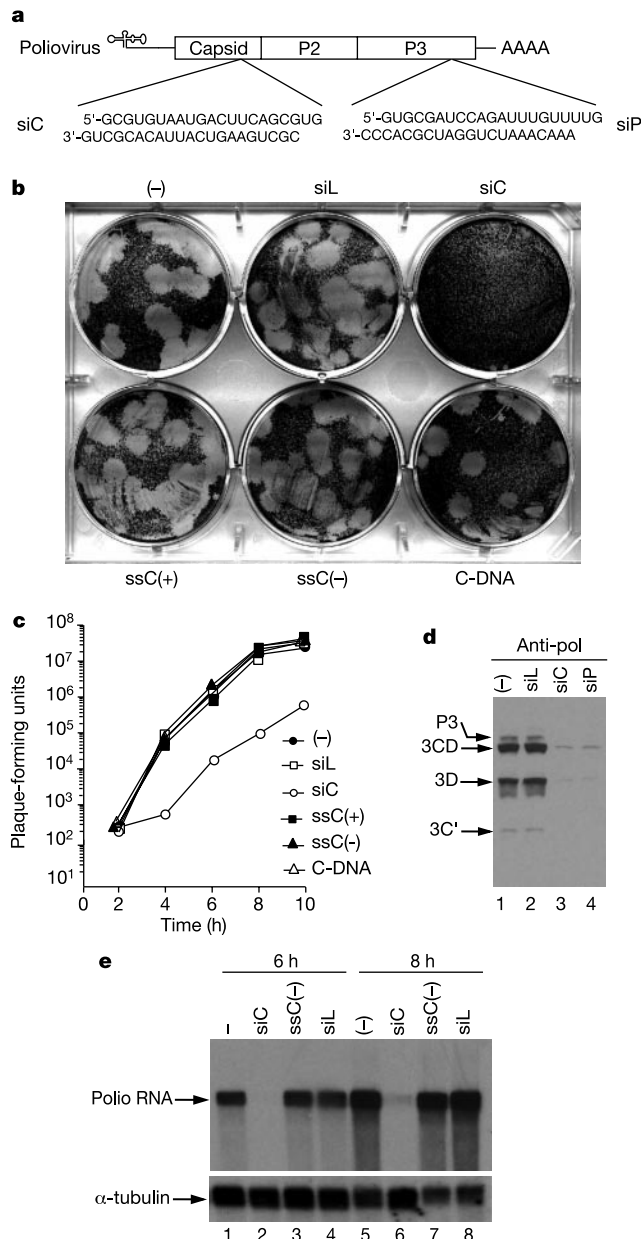


Figure 1 Poliovirus growth suppressed by siRNA. **a**, Schematic representation of poliovirus genome and relative locations of siC and siP. **b**, Plaque reduction assays: HeLa monolayers were transfected with siC or controls. After removing transfection mixtures, monolayers were infected with poliovirus (approximately 30 plaque-forming units). **c**, One-step poliovirus growth curve using cells pre-treated with siRNA. **d**, Western blot analysis with anti-3D^{pol} of cells transfected with buffer (lane 1), siL (lane 2), siC (lane 3) or siP (lane 4) and infected with poliovirus (MOI = 10). **e**, Northern blot analysis of poliovirus RNA at 6 and 8 h after infection; cells were pre-transfected with siRNA as indicated. α -tubulin served as the loading control.

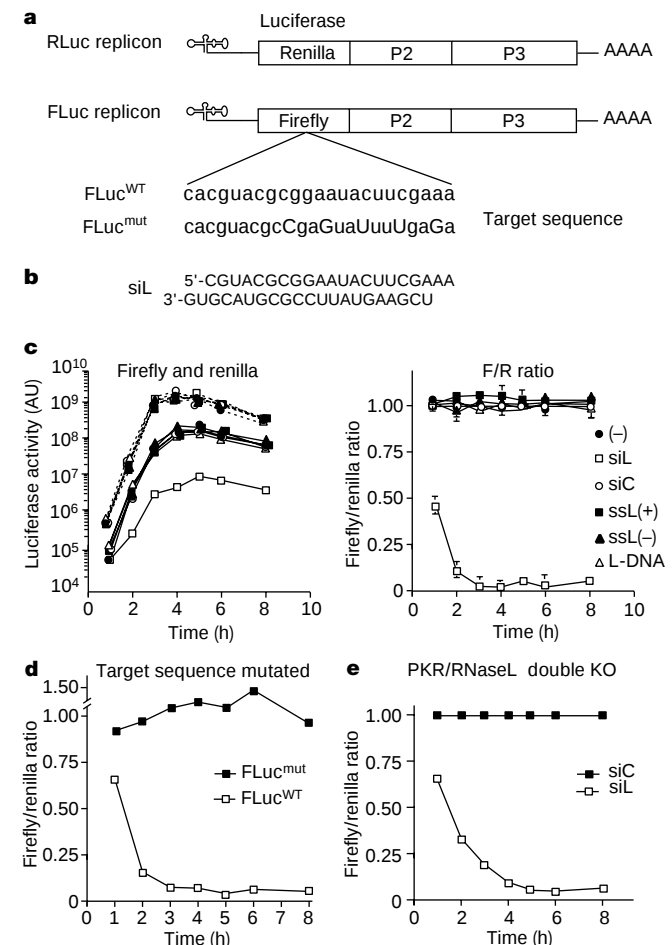


Figure 2 siRNA inhibits replication of poliovirus replicon in a sequence-dependent manner. **a**, Schematic representation of poliovirus firefly and renilla luciferase replicons. The target sequences in FLuc^{WT} and FLuc^{mut} are shown, where uppercase letters indicate a point mutation. **b**, Firefly luciferase-specific siRNA. **c**, Firefly (solid lines) and renilla (dotted lines) luciferase activity of replicons co-electroporated into HeLa cells with siRNAs or L-DNA. The right panel represents firefly/renilla ratios normalized to siC, (at each time point siC = 1.0); this data is the mean ratio values for three experiments $\pm$ s.d. AU, arbitrary units. **d**, Firefly luciferase expression by mutant (FLuc^{mut}) and wild-type (FLuc^{WT}) replicons in siL-transfected HeLa cells, normalized as in **c**. **e**, Inhibition of firefly luciferase production by siL in PKR/RNaseL double-knockout cells, normalized as in **c**.

transfection of replicons with siL resulted in a specific reduction of firefly, but not renilla, luciferase produced (Fig. 2c). Conversely, siC, which inhibits viral production (Fig. 1), did not affect FLuc luciferase expression and replicon RNA replication (Fig. 2c). Furthermore, siL had no inhibitory effect on FLuc^{mut}, a firefly luciferase replicon bearing five silent point mutations within the siL target sequence (Fig. 2a, d). Thus, the inhibitory effect requires a close match between the siRNA and the target RNA sequences.

It is, in principle, possible that inhibition of viral replication is caused by an interferon-mediated response, which can be induced by dsRNA¹⁵. We thus examined whether interferon or other secreted factors mediate the siRNA-induced inhibition, and found that supernatants from siC-transfected cells have no effect on poliovirus replication (not shown). Furthermore, dsRNA activates two interferon-induced enzymes, PKR and RNaseL, which inhibit gene expression in a nonspecific manner. These two proteins have also been suggested to participate in activation of sequence-specific gene silencing systems^{16,17}. However, a robust inhibition of FLuc expression was observed in response to siL in mouse fibroblasts deficient in PKR and RNaseL (PKR/RNaseL double knockout) (Fig. 2e). We conclude that neither interferon nor its dsRNA-activated effectors PKR and RNaseL is required for suppression of poliovirus replication by siRNA.

The siRNA-mediated inhibition of poliovirus replication must involve mammalian post-transcriptional gene silencing (PTGS)

mechanisms, as the virus replicates in the cytoplasm through RNA intermediates. There are three possible modes for this PTGS action: it may interfere with viral RNA synthesis, block translation of the viral genome as observed for small temporal RNAs¹⁸, or destabilize the viral RNA, as observed for RNAi in *Drosophila* extracts¹⁹. Because a non-replicating RNA expressing firefly luciferase (FLuc messenger RNA, Fig. 3a) is also susceptible to inhibition by siL (Fig. 3b), we concluded that RNA replication is not required

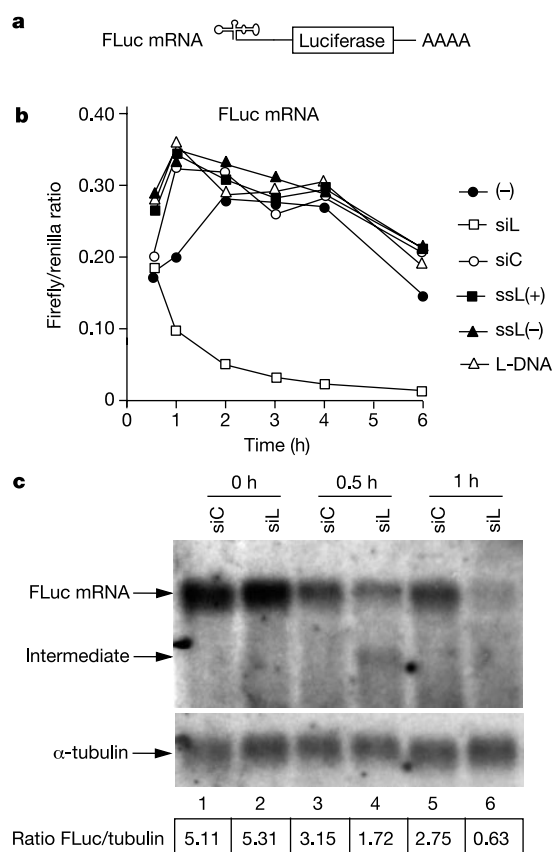


Figure 3 Gene silencing is rapid, post-transcriptional, and not dependent on viral replication. **a**, Schematic representation of non-replicating firefly luciferase mRNA (FLuc mRNA). **b**, FLuc mRNA was electroporated into cells together with various siRNAs. Results are shown as firefly/renilla ratios. **c**, Northern blot of firefly luciferase mRNA electroporated with siC or siL at various times after infection. The lower arrow indicates a putative 3' cleavage intermediate derived from Luc mRNA. α -tubulin served as the loading control. The amount of radioactivity in each band was determined by phosphorimaging. Ratios of FLuc to tubulin mRNA are indicated at the bottom.

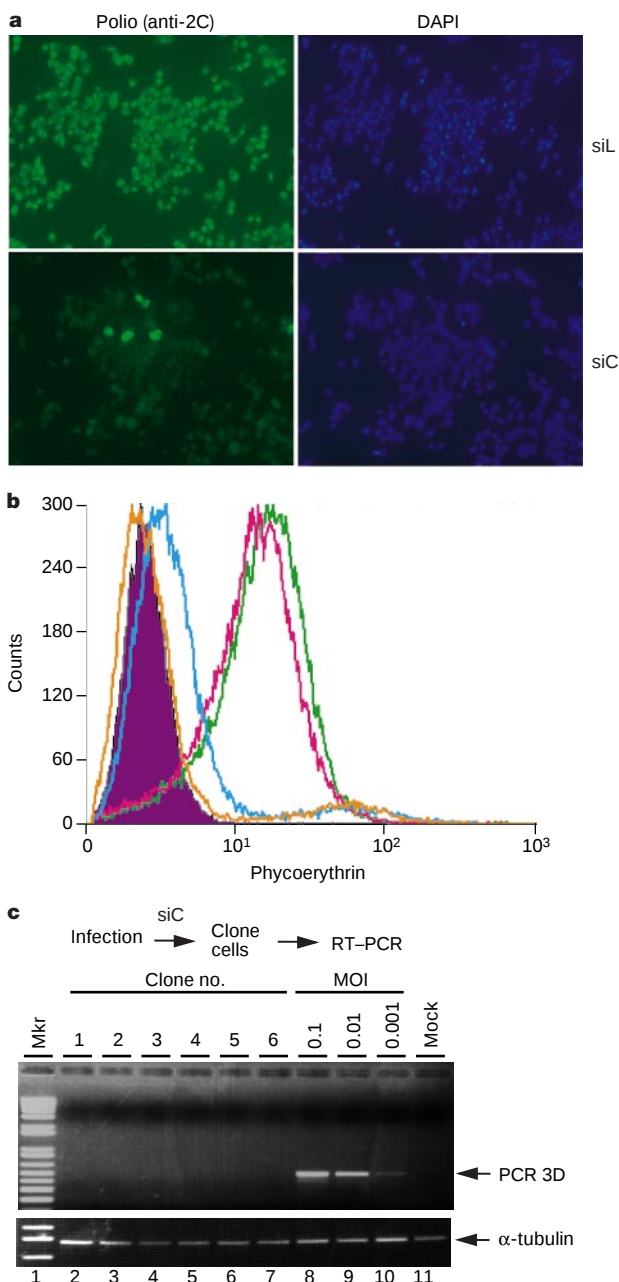


Figure 4 Most cells treated with siC clear infection and do not harbour viral genomes. **a**, Immunofluorescence of siL/siC-treated cells at 6 h after infection, infected at MOI = 10. The poliovirus-infected cells were distinguished using an anti-2C antibody, and nuclei were stained by DAPI. **b**, FACS profile of cells transfected with buffer (red), siL (green), siC (orange), or siP (blue), then infected (MOI = 10). Control uninfected cells are indicated by the solid purple section. **c**, RT-PCR of viral RNA from six independent cell clones obtained from infected, siC-transfected cultures (lanes 2–7). For a positive control, RT-PCR cells were infected at a low MOI (0.1, 0.01, 0.001) for 1 h (lanes 8–10). At a MOI of 0.001, fewer than 1,000 cells are expected to be infected, indicating that the assay is very sensitive. α -tubulin served as a loading control. Mkr, marker.

for siRNA inhibition. We then examined the stability of FLuc mRNA in cells transfected with either siC or siL (Fig. 3c). Treatment with siL induced a reduction in the levels of FLuc mRNA, and this effect was most evident 1 h after transfection (Fig. 3c; compare lanes 5 and 6). Notably, a weak band corresponding in size to the expected 3' cleavage product, presumably an intermediate of degradation, was detectable at 0.5 h after transfection (Fig. 3c, lane 4). These experiments indicate that RNA interference by siRNAs in mammalian cells is mediated, at least in part, by mRNA degradation.

The 100-fold reduction in viral titre caused by siRNA treatment could result from a reduction in virus production per infected cell or alternatively from a reduction in the number of productively infected cells. As the high MOI used in our experiments ensures that all cells are infected, the latter possibility would indicate that gene silencing is capable of aborting viral infection. The number of cells expressing viral proteins at 6 h after infection was determined by immunofluorescent staining and fluorescence-activated cell sorting (FACS) analysis. Notably, over 90% of cells did not express detectable amounts of viral proteins (Fig. 4a, b).

siRNA-treated cells, negative for viral antigen, may be completely cleared of infection or may carry viral genomes expressing low amounts of viral proteins. Thus, we cloned, by limiting dilution, cells infected with polio (MOI = 10) and treated with siC to detect the presence of latent virus. In contrast to controls, siC- and siP-transfected cells reproducibly formed colonies in at least half of the wells (not shown). Importantly, we were unable to re-isolate virus from these cultures, and a highly sensitive polymerase chain reaction with reverse transcription (RT-PCR) analysis failed to detect viral RNA, indicating that the cloned cells are not persistently infected (Fig. 4c). Although protected from the original infection, the cloned cells were fully susceptible to reinfection with poliovirus 21 days after siRNA treatment (not shown). Taken together, these data indicate that dsRNA can induce effective antiviral intracellular immunity that clears most cells of infection.

We next evaluated the protective effect of siRNAs over a time course of poliovirus infection. At 24 h after infection, cells transfected with siC before infection show no signs of cytopathic effect,

whereas control cultures are completely lysed by 8 h (Fig. 5a). About 75% of siC- or siP-transfected cells were viable 26 h after infection; notably, a combination of siC + siP extended the protection to 54 h (Fig. 5b). However, despite the initial protective effect, prolonged incubation led to cell lysis, even after siC RNA treatment (Fig. 5b). We thus considered two possible reasons for loss of protection: waning of siRNA activity or emergence of siRNA-resistant viruses.

To address these possibilities, cells were treated for 24 h with siC or siP and infected with poliovirus at 0, 1, 2, 4 or 5 days after siRNA treatment. Effective inhibition of viral production was observed as late as 5 days after transfection (Fig. 5c). Thus, waning of siRNA activity over time does not seem to account for the observed monolayer destruction. However, the virus isolated from siC-transfected cells was barely, if at all, sensitive to siC (not shown). Sequencing analysis revealed silent point mutations in most of the clones analysed (19 clones in total). There were two types of siC-resistant mutants (siC^r-1 and siC^r-2) carrying U → C transitions in the middle of the siC target region (Fig. 5d). Thus, it seems that a small sequence divergence allowed escape from protection through siRNA. These results underscore the importance of sequence identity of siRNA to the target sequence for effective antiviral activity. Notably, these escape mutants rarely emerged when cells were infected with lower MOIs (not shown). This, together with the fact that many infected cells are completely free of viral RNA (Fig. 4c) suggests that the siRNA-resistant variants were present in the initial viral population and did not emerge from the siRNA-treated cells. We conclude that the PTGS apparatus interacts directly with viral sequences and is not dependent on activation of other, intermediary antiviral mechanisms.

The present work indicates that the mammalian PTGS machinery can be programmed with siRNAs corresponding to viral sequences to induce an effective antiviral response. It is possible that, as observed in plants, gene silencing functions as an adaptive, nucleic-acid-based defence system in mammalian cells. However, additional experiments are necessary to determine whether PTGS mechanisms function as a natural intracellular response to viral infection, without the need for pre-treatment with synthetic

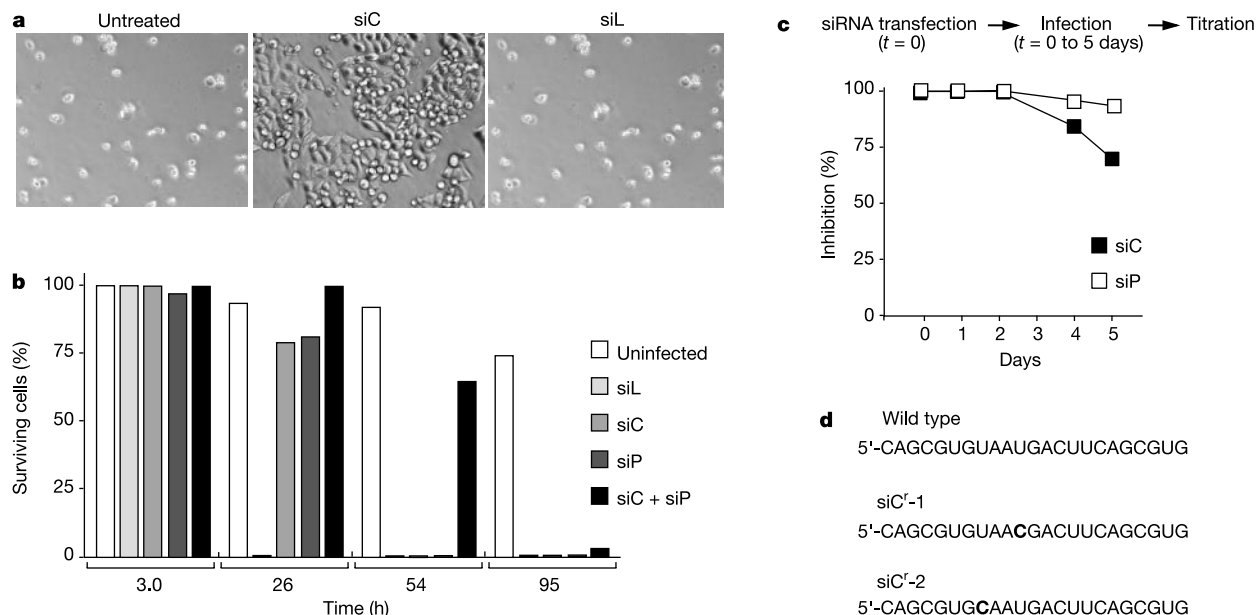


Figure 5 Infected cells survive more than a day, but are eventually lysed by emerging siRNA-resistant virus. **a**, Bright-field micrographs of cells pre-transfected with buffer, siC and siL, taken 24 h after infection (MOI = 10). **b**, Cell viability in the presence of siRNA, determined by trypan blue staining at various times after infection. **c**, HeLa cells were transfected with siRNAs (siL, siC and siP) and infected 0, 1, 2, 4 or 5 days after

transfection (scheme). Virus titres were determined 24 h after infection. The effect of siC and siP is presented as the per cent titre reduction with respect to siL negative control (0% reduction). **d**, siC target sequences of sensitive (wild type) and siC-resistant (siC^r) virus genomic RNAs.

siRNAs. Even though our results indicate that poliovirus is susceptible to siRNA-mediated gene silencing, it is possible that the virus neutralizes the PTGS pathway by, for example, inhibiting the processing of long, viral dsRNA to siRNA. Our demonstration of viral genome clearance from mammalian cells infected by an otherwise lytic virus is evidence that sterilizing antiviral immunity may be achieved without the destruction of cells harbouring virus.

It was previously proposed that intracellular immunization might be achieved by expression of proteins or RNAs that confer resistance to virus infection²⁰. Double-stranded RNA is a promising vehicle for induction of intracellular immunity. Unlike classical antisense techniques, double-stranded RNA taps into existing, powerful gene silencing pathways, which may facilitate its therapeutic potential. Our results also predict that attempts to use siRNA therapeutically against viruses, especially RNA viruses, will have to contend with their variability due to high mutation rates; however, the emergence of escape variants resistant to siRNA can be minimized by using dsRNA directed against multiple conserved RNA target sequences. In addition, this model system can be exploited to further examine the mechanism of action of PTGS in mammalian cells and the ability of RNA viruses to neutralize the inhibitory effect. □

Methods

Molecular biology procedures

RNA oligonucleotides were purchased from Dharmacon Research, and resuspended in 1 mM sodium citrate at 100 μ M. For generation of double-stranded siRNA, individual strands were mixed, heated to 100 °C for 3 min and allowed to cool to room temperature; final concentration of the siRNAs was 40 μ M in 100 mM potassium acetate, 30 mM HEPES, pH 7.4, 2 mM magnesium acetate. DNA oligonucleotides were purchased from Operon Technologies, and treated similarly where used as siRNA controls. Cytoplasmic RNA was isolated from cells in accordance with RNeasy protocol (Qiagen). Reverse transcriptions were done with SuperScript RT (Invitrogen) using oligonucleotide d(T) primer. PCRs were run with Elongase (Invitrogen) for detection of viral/tubulin- α RNA or PfuTurbo (Stratagene) for amplification and cloning of complementary DNA derived from resistant viruses, according to manufacturers' instructions. PCR primers used were: tubulin- α : 5'-GATGGAGCCCTGAATGTTGA-3' and 5'-TGATGTTAATGACITTTACTTTGAGATATG-3'; Mahoney detection: 5'-TATGATGCATCTCTCAGCCCT-3' and 5'-GCGAA CGTGATCCTGAGTGTT-3'; Mahoney cloning: 5'-GCTAGACACCGTGTCTTGA-3' and 5'-GGACTGTGTGTCAATCATGCT-3'.

Northern blotting hybridizations were done at 42 °C in 50% formamide as described²¹, with probes prepared using RediPrime kit (Amersham-Pharmacia). The probes corresponded to nucleotides 1894–2408 of the poliovirus genome, 1109–1601 of the luciferase coding region, and 732–1385 of α -tubulin coding region.

Western blotting was performed by standard protocols with ECL System reagents (Amersham-Pharmacia), using purified rabbit polyclonal antibody directed against a 3D amino-terminal peptide. Cells were grown in 24-well plates, lysed in 50 μ l of 1% NP40 in 140 mM NaCl, 5 mM KCl, 10 mM Tris, pH 7.5, and 5 μ l of each sample loaded on a denaturing 12% polyacrylamide gel.

Cell culture and virus

HeLa S3 and mouse embryonic fibroblasts were cultured in DMEM/F12 supplemented with 10% FBS, 2 mM glutamine, 100 U ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin (Invitrogen).

Immortalized PKR/RNaseL-deficient cells were established from mouse embryonic fibroblasts²² by transfecting them with a plasmid coding for SV40 large T-antigen. Typically, cells were seeded at 5×10^4 per well the previous day, and transfected with 2 μ l LipofectAMINE 2000 (Invitrogen) combined with 3 μ l of 40 μ M siRNA. Transfection mixtures were left on cells overnight and washed off immediately before viral infections, unless indicated otherwise.

Mahoney strain of poliovirus was produced from a cDNA clone by T7 transcription and amplified in HeLa S3 cells. For infections, virus was diluted in PBS or culture medium and adsorbed onto cells for 20–40 min. Incubations were at 37 °C. For plaque reduction assays, the monolayers were covered with 1% semi-solid agar overlay and stained with crystal violet after 2 days. For titre determination, cells were collected from the wells, lysated by three freeze-thaw cycles, and the virus titred by plaque assays²³.

RNA electroporations were done essentially as described²⁴, but were modified to contain 10 μ g of 1:1 firefly:renilla replicon mixture $\pm$ 5 μ l of 40 μ M siRNA; for PKR/RNaseL knockouts, approximately 4×10^6 cells were used per electroporation. Luciferase assays were performed using the Dual Luciferase Assay System (Reporter kit, Promega).

Microscopy, immunofluorescence and FACS

Cells were observed on a Nikon Eclipse TE2000 microscope and photographed using a Spot CCD (charge-coupled device) camera. For immunofluorescence, cells were grown on coverslips and fixed in 4% PFA for 20 min at room temperature, then washed in PBS,

permeabilized with 0.1% TritonX-100, and stained with anti-N-terminal poliovirus 2C rabbit antiserum diluted 1:500 into 1% BSA/PBS. Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody was used (Molecular Probes) at 1:300, and coverslips were mounted in VectaShield medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector). Pictures were taken on a Leica DMLB fluorescent microscope with ScionImage software. For FACS analysis, cells were permeabilized with 0.02% saponin and stained with 1:1,500 dilution of anti-carboxy terminal 2C rabbit serum, then treated with phycoerythrin-conjugated donkey anti-rabbit secondary antibody (Jackson Labs). FACS was performed with 25,000 cells per condition, using FACSCalibur and CellQuest software (Becton-Dickinson).

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Competing interests statement

The authors declare that they have no competing financial interests.

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Modulation of HIV-1 replication by RNA interference

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RNA interference (RNAi) is the process by which double-stranded RNA (dsRNA) directs sequence-specific degradation of messenger RNA in animal and plant cells^{1,2}. In mammalian cells, RNAi can be triggered by 21-nucleotide duplexes of small interfering RNA (siRNA)³. Here we describe inhibition of early and late steps of HIV-1 replication in human cell lines and primary lymphocytes by siRNAs targeted to various regions of the HIV-1 genome. We demonstrate that synthetic siRNA duplexes or plasmid-derived siRNAs inhibit HIV-1 infection by specifically degrading genomic HIV-1 RNA, thereby preventing formation of viral complementary-DNA intermediates. These results demonstrate the utility of RNAi for modulating the HIV replication cycle and provide evidence that genomic HIV-1 RNA, as it exists within a nucleoprotein reverse-transcription complex, is amenable to siRNA-mediated degradation.

RNAi is a ubiquitous mechanism of gene regulation in plants and animals⁴ in which target mRNAs are degraded in a sequence-specific

manner⁵. RNAi is initiated by the dsRNA-specific endonuclease Dicer, which promotes processive cleavage of long dsRNA into double-stranded fragments between 21 and 25 nucleotides long, termed siRNAs⁵⁻⁸. Small interfering RNAs are incorporated into a protein complex that recognizes and cleaves target mRNAs⁹. Introduction of dsRNA into mammalian cells does not result in efficient Dicer-mediated generation of siRNA and therefore does not induce RNAi^{10,11}. The requirement for Dicer in maturation of siRNAs can be bypassed by introducing synthetic 21-nucleotide siRNA duplexes, which inhibits expression of transfected and endogenous genes in a variety of mammalian cells³. HIV-1 uses RNA intermediates in its replication. Therefore, we examined whether siRNA duplexes specific for HIV-1 were capable of effecting the degradation of viral RNAs necessary for completion of early and late events in the viral replication cycle.

We directed 21-nucleotide siRNA duplexes against several regions of the HIV-1 genome, including the viral long terminal repeat (LTR) and the accessory genes *vif* and *nef* (Fig. 1a). Small interfering RNA duplexes were co-transfected with an HIV-1 molecular clone (HIV_{NL-GFP}; ref. 12) into CD4-positive HeLa (Magi) cells¹³. Transfection of cells with an infectious molecular HIV-1 clone recapitulates late events in the viral life cycle, including production of viral RNAs, translation of viral proteins and release of virions. Compared with cells not transfected with siRNA duplexes, virus production, measured 24 h after transfection, was reduced 30-fold to 50-fold by homologous siRNAs (Fig. 1b). HIV production was inhibited to a lesser extent by single mismatch siRNAs (MTAR, M441), whereas a

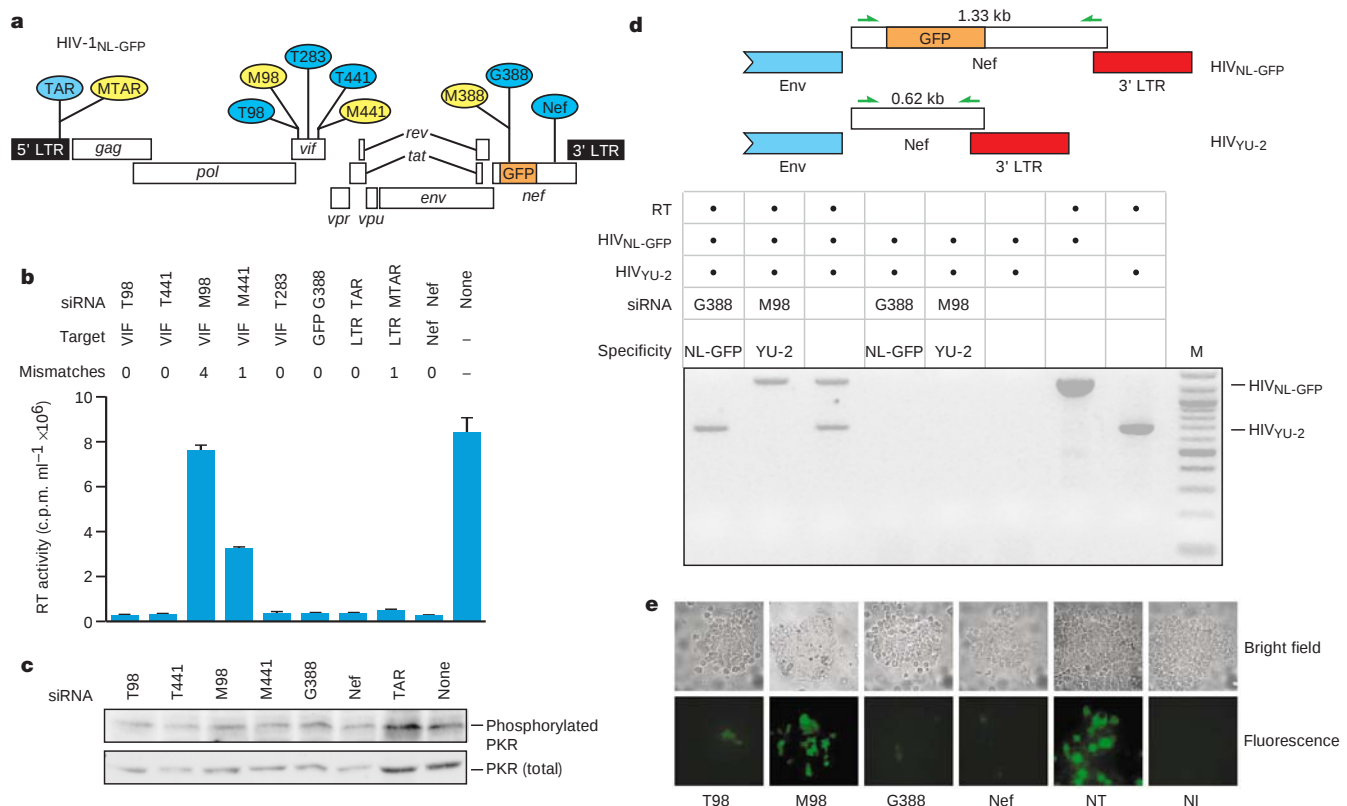


Figure 1 Small interfering RNAs inhibit late events in HIV replication by promoting degradation of HIV-1 RNA. **a**, HIV targets of siRNAs used in this study. Small interfering RNAs completely homologous to the target HIV sequence (HIV_{NL-GFP}) are shown in blue and those harbouring nucleotide mismatches are shown in yellow. **b**, Effect of siRNAs on HIV-1 particle production. **c**, Total and active (phosphorylated) PKR levels in siRNA-transfected Magi cells. **d**, Small interfering RNAs mediate sequence-specific HIV RNA

degradation. The presence of HIV_{NL-GFP} or HIV_{YU-2} RNA was determined by RT-PCR using HIV Nef-specific primers. Because of the GFP insertion in HIV_{NL-GFP} Nef, RNAs originating from HIV_{NL-GFP} are 710 nucleotides larger than those originating from HIV_{YU-2}. M, molecular weight marker (100 bp ladder, New England Biolabs). **e**, Effect of siRNAs on HIV expression in primary PBLs.

vif siRNA with four mismatches (M98) did not inhibit HIV production (Fig. 1b). Activation of the dsRNA-activated protein kinase PKR leads to an inhibition of protein translation in a sequence-non-specific manner relative to the inducing dsRNA. Activation with PKR was not involved in the inhibition of the negative-strand RNA virus RSV (respiratory syncytial virus) by siRNAs¹⁴. Similarly, there was no significant induction of activated PKR (phosphorylated on Thr 446) over levels in non-transfected cells by any of the siRNAs (Fig. 1c). To further exclude a PKR effect, Magi cells were co-transfected with two HIV-1 variants (HIV-1_{NL-GFP}, HIV-1_{YU-2}; ref. 15) and with siRNAs that are specifically targeted to either virus. Because of the presence of a green fluorescent protein (GFP) insertion in Nef, HIV_{NL-GFP} should be targeted by the GFP-specific siRNA G388, whereas HIV_{YU-2}, which lacks a GFP insert, should be insensitive to G388. In addition, we exploited sequence differences in the *vif* genes of these viruses. The M98 siRNA contains four mismatches relative to the HIV_{NL-GFP} *vif* gene but is completely homologous to HIV_{YU-2} *vif*. Thus, M98 should direct the specific inhibition of HIV_{YU-2} RNA and not HIV_{NL-GFP} RNA. Because of the GFP insertion in HIV_{NL-GFP}, viral RNA produced in cells harbouring both viruses could be distinguished. In the absence of siRNAs, both HIV_{NL-GFP} and HIV_{YU-2} RNAs were evident in co-transfected cells (Fig. 1d). However, co-transfection with the G388 siRNA resulted in a loss of HIV_{NL-GFP} RNA but not HIV_{YU-2} RNA. Conversely, the M98 siRNA caused a loss in HIV_{YU-2} RNA without affecting HIV_{NL-GFP} RNA (Fig. 1d). This sequence-specific inhibition is inconsistent with a sequence-non-specific PKR effect and indicates that siRNAs are inhibiting HIV production by causing the specific degradation of viral RNA. We next examined

whether siRNAs could inhibit HIV gene expression (GFP fluorescence) in primary peripheral blood lymphocytes (PBLs), which are natural targets for HIV-1 infection. The frequency of GFP-expressing cells was markedly reduced in cells transfected with homologous siRNAs (T98, G388, *nef*) relative to cells transfected with mismatched siRNAs or non-transfected cells (Fig. 1e). The level of HIV_{NL-GFP} RNA, as determined by polymerase chain reaction with reverse transcription (RT-PCR), was also markedly reduced in cells transfected with homologous siRNAs (results not shown). Therefore, the components of siRNA-activated RNAi are fully functional in cells naturally targeted by HIV-1 infection.

Upon HIV-1 infection, genomic viral RNA is introduced into the host cell cytoplasm in the form of a nucleoprotein complex, which comprises viral proteins in association with genomic viral RNA¹⁶. Within this complex, the viral reverse transcriptase enzyme directs the synthesis of viral cDNA intermediates from the genomic viral RNA template. Recent studies with RSV have indicated that genomic viral RNA, which is tightly associated with nucleocapsid protein, is resistant to siRNAs¹⁷. We investigated whether siRNAs were able to direct the specific degradation of genomic viral RNA of HIV-1. The experimental design is outlined in Fig. 2a. Magi cells were transfected with the various siRNAs and infected with HIV_{NL-GFP} 20 h later. Transfection of cells with siRNAs did not significantly interfere with virus uptake *per se*, on the basis of levels of cell-associated p24 at 1 h after infection (Fig. 2b). The strategy for analysis of viral reverse-transcription intermediates in acutely infected cells is outlined in Fig. 2c. At 1 h after infection, genomic viral RNA was specifically detected in cells transfected with mismatched siRNAs and in non-transfected cells (M98, M441) but not

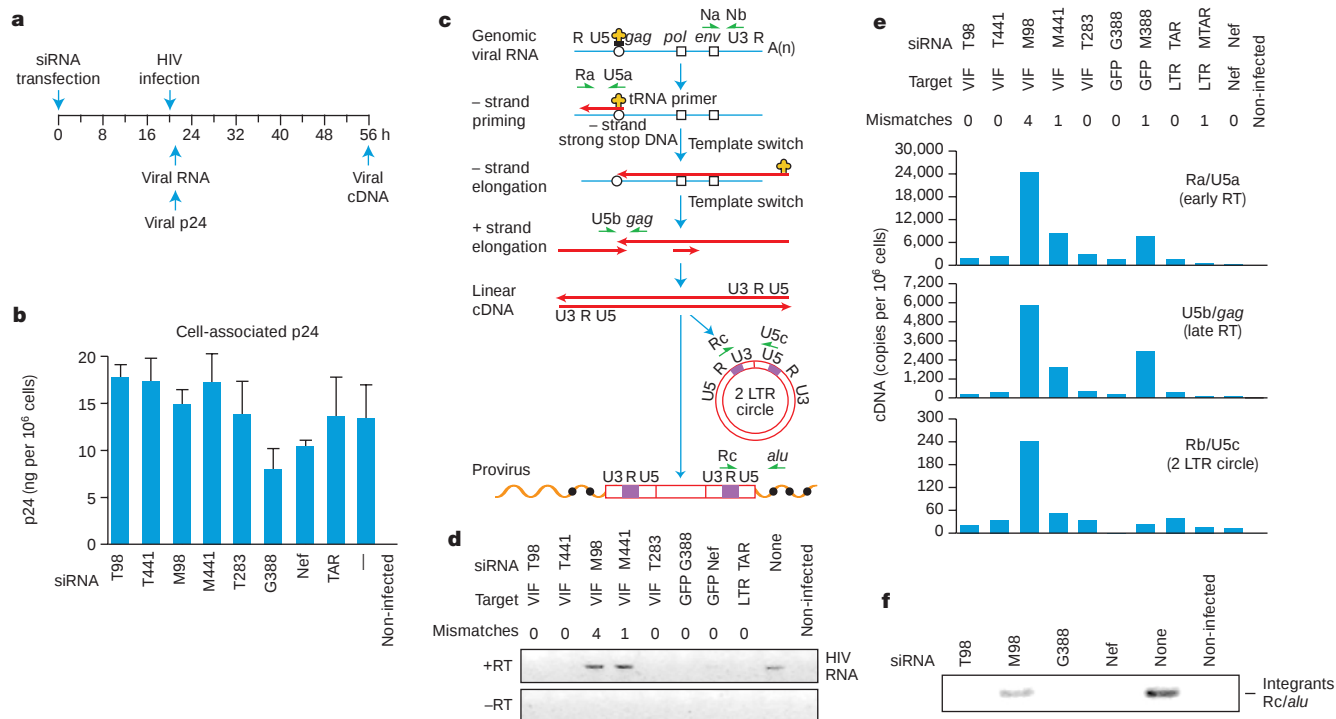


Figure 2 Small interfering RNAs block early events in HIV replication by promoting degradation of genomic HIV RNA. **a**, Experimental design. **b**, Levels of trypsin-resistant HIV *gag* p24 in siRNA-transfected cells. Dash indicates no siRNA transfected into the cells. **c**, Strategy for analysis of viral nucleic acid intermediates formed early after HIV infection. Major cDNA intermediates in viral reverse transcription are indicated. Blue line, viral RNA; red line, viral cDNA; open circles and squares, primer-binding sites for initiation of minus-strand synthesis and polypurine tracts for plus-strand synthesis, respectively. HIV-specific

primers (green arrows) are shown next to the earliest cDNA intermediate they amplify. Integrated (proviral) HIV DNA was amplified using an HIV LTR-specific primer (Rc) and a primer directed to *alu* repeats (filled circles) within flanking cellular DNA. **d**, Effect of siRNAs on genomic viral RNA. **e**, Effect of siRNAs on formation of HIV-1 reverse transcription (RT) intermediates. **f**, Reduced levels of viral integration in siRNA-transfected cells.

in cells transfected with homologous siRNAs (Fig. 2d). Because genomic viral RNA is the template for the synthesis of viral cDNA intermediates, the synthesis of viral cDNAs, determined 36 h after infection, was dramatically inhibited in cells transfected with homologous siRNAs (T98, GFP, *nef*) (Fig. 2e). Small interfering RNAs bearing one-nucleotide mismatches (M441, M388) were partially inhibitory relative to the siRNA bearing four mismatches (Fig. 2e). Small interfering RNAs were quite stable in cells: HIV entry was suppressed to equal levels whether virus was added 20 h or 4 days after siRNA transfection (data not shown). Upon completion of viral cDNA synthesis, viral sequences integrate into cellular DNA to form a provirus. The level of provirus formation, as evidenced by the presence of junction sequences flanking viral and cellular DNA (Fig. 2e), was markedly reduced in cells transfected with homologous siRNAs (T98, G388, *nef*) relative to cells transfected with mismatched (M98) siRNAs or non-transfected cells (Fig. 2f). Collectively, these studies indicate that siRNAs interrupt early events in the HIV replication cycle by directing the specific degradation of genomic HIV-1 RNA, thereby preventing the subsequent synthesis of viral reverse-transcription intermediates and establishment of the provirus.

Expression of siRNAs from plasmid templates offers several advantages over synthetic siRNAs, such as stable selection under

selectable markers and inducible promoters, which are features that could be useful for genetic approaches to HIV therapy. Therefore, we examined whether expressed siRNAs could inhibit HIV. Modifying a strategy used previously in plants^{18,19}, we constructed plasmids containing a 19-base pair (bp) region of the HIV-1 *vif* gene in 5'-3' and 3'-5' orientations under the control of a T7 promoter (Fig. 3a). Virus production was determined 24 h after a three-way transfection of Magi cells with an HIV_{NL}-GFP molecular clone, the linearized *Vif* hairpin plasmid (TL *Vif*) and a vector expressing T7 RNA polymerase (T7 Pol). In the presence of T7 RNA polymerase, T7 transcripts derived from *Bst*BI-linearized expression plasmids would be predicted to comprise a GGUACC sequence from the T7 promoter, a 19-bp stem of self-complementary *Vif* sequences, a 3-, 5- or 7-nucleotide loop and a 3' UU overhang. All three *Vif* hairpin plasmids containing 3-, 5- or 7- nucleotide loops potentially suppressed virus production to 20–30-fold relative to non-transfected cells. By comparison, the presence of an identical plasmid lacking *Vif* sequences (TL Δ *Vif* or a control plasmid pcDNA) had no effect on virus production in co-transfected cells (Fig. 3b). This inhibitory effect on virus production was reflected by a loss of viral RNA (Fig. 3c). The *Vif* hairpin plasmid (TL *Vif*7) also inhibited viral gene expression in primary lymphocytes, whereas there was no inhibitory effect of the plasmid lacking *Vif* sequences in these cells (Fig. 3d). These results indicate that a sequence-specific RNAi effect can be activated in established and primary cells by siRNAs derived from self-complementary hairpin-generating plasmids. This provides a rationale for gene-therapy approaches to HIV that complement existing post-transcriptional approaches for inhibiting HIV, including ribozymes and antisense RNA (reviewed in ref. 20).

The involvement of RNAi in transposon silencing^{21,22} suggests that RNAi is an ancient antiviral system that may have evolved as a defence mechanism to protect the host from invasion by mobile genetic elements including transposons and viruses. Several studies have indicated that it is difficult to induce RNAi in mammalian cells using long dsRNAs. Although long dsRNAs can modestly inhibit gene expression in mammalian cells, the effects are not sequence specific^{3,23} and are more consistent with inhibition by the interferon response. Intriguingly, it is now becoming apparent that underlying the non-specific dsRNA-activated interferon response in mammalian cells, there may indeed be a sequence-specific RNAi effect that can be activated by long dsRNA^{24–26}. Silencing by long dsRNAs has now been observed in various cultured mammalian cells^{24,25}. The mechanism of silencing is consistent with RNAi because there is evidence that the long dsRNAs are processed to siRNAs and target RNAs are specifically degraded. Our results indicate that 21-nucleotide siRNAs promote HIV RNA degradation in primary lymphocytes, suggesting that the major target cell for HIV replication possesses functional components of the siRNA-induced silencing complex that mediates specific cleavage of target RNA². Future studies should determine whether sequence-specific RNAi that is independent of the interferon response can be activated against HIV by long dsRNAs. □

Methods

Synthesis of siRNA

The following RNA oligonucleotides were purchased from Dharmacon: T98 (5'-GGAAAGCUAAGGACUGGUUdTdT-3'); T283 (5'-AGCACACAAGUAGACCCUGdTdT-3'); T441:5' -CUUGGCACUAGCAGCAUUAdTdT-3'); M98 (5' GAAAGCUAGGGGAUGGUUdTdT-3'); M441 (5' -CUUGGCACUAGCAGCAUUAdTdT-3'); G388 (5' -GACUUAUAGGAGUAGGCAUdTdT-3'); M388 (5' -GACUUAUAGGAGUAGGCAUdTdT-3'); *nef* (5' -GUGCCUGGCUAGAAGCACdTdT-3'); TAR (5' -AGACCAGAUUCUGAGCCUGdTdT-3'); and MTAR (5' -AGACCAGAUUAUGAG CCUGdTdT-3').

Plasmids

The T7 promoter was modified in the plasmid PCRscript (Stratagene) to form pCRT7. Oligonucleotides corresponding to nucleotides 5,323–5,342 of HIV-1 *vif* (Genbank accession number M19921) were inserted at the *Srf*I site of pCRT7. T7 Pol comprises T7 RNA polymerase from *Escherichia coli* BL21 (DE3) cloned into pcDNA 3.1 (Invitrogen).

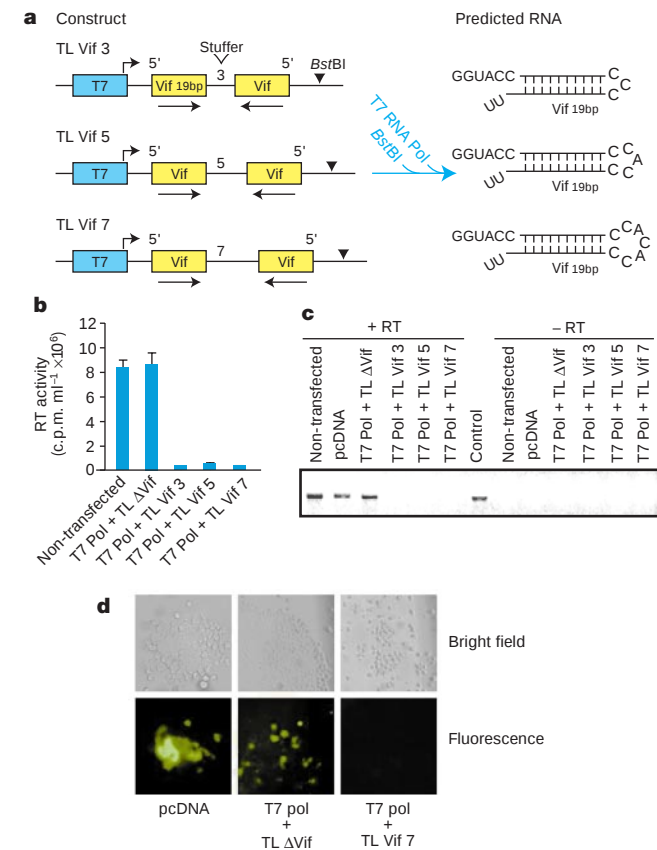


Figure 3 Inhibition of HIV replication by siRNAs derived from plasmid DNA templates. **a**, Strategy for production of hairpin siRNAs from plasmid vectors. Linearization of each construct with *Bst*BI and transfection into cells with a plasmid expressing T7 RNA polymerase (Pol) predicts the expression of a hairpin RNA with a 19-bp self-complementary *Vif* stem and non-base-paired loops of 3, 5 and 7 nucleotides. **b**, Effect of *Vif* hairpin siRNAs on HIV particle production. TL Δ *Vif* is identical to plasmids that express *Vif* hairpin except that it lacks self-complementary *Vif* sequences. **c**, *Vif* hairpin siRNAs promote degradation of HIV RNA. PCR products amplified from HIV_{NL}-GFP served as a control. **d**, Inhibition of HIV-1 expression by *Vif* hairpin siRNAs in primary PBLs.

Cells and transfections

Magi cells were grown in DMEM containing 10% fetal bovine serum (FBS). PHA-activated, elutriated PBLs were cultured in RPMI containing 10% FBS and $64 \mu\text{ml}^{-1}$ of interleukin-2 (ICN). Magi cells were transfected with oligofectamine (GIBCO) by the manufacturer's protocol in the presence of $1 \mu\text{g}$ HIV plasmid and/or 60 pmol of siRNA oligonucleotides. Transfection efficiencies were 75–85%. For PHA-activated PBLs, 5×10^6 cells were electroporated using a Gene Pulser apparatus (Bio-Rad) at 250 V , $960 \mu\text{F}$, resistance $R = \infty$ with $5 \mu\text{g}$ plasmid and/or 200 pmol siRNA. Transfection efficiencies were 30–50% of viable cells. Three-way transfections with siRNA expression plasmids comprised $0.1 \mu\text{g}$ T7 Pol, $0.5 \mu\text{g}$ pTLVif and $0.5 \mu\text{g}$ pNLGFP (Magi cells), or $0.5 \mu\text{g}$ T7 Pol, $2 \mu\text{g}$ TLVif and $2 \mu\text{g}$ pNLGFP (for primary lymphocytes). Transfected cells were centrifuged (1,200g) on DAKO silanized slides and examined under bright-field illumination or fluorescence (wavelength 516 nm) on a Zeiss Axioplan 2 microscope.

PCR analysis

Real-time PCR was performed as previously reported²⁷. Products were amplified from 5 to $20 \mu\text{l}$ of extrachromosomal DNA in $50\text{-}\mu\text{l}$ reactions containing $1 \times$ HotStart Taq buffer (Qiagen), 200 nM dNTPs, 400 nM primers and 1.5 U HotStart Taq. Two-LTR junctions were amplified by the primers Rc ($5'$ -TAGACCAGATCT GAGCCTGGGA- $3'$) and U5c ($5'$ -GTAGTCTTGCCCAATCAGGG AAG- $3'$). Early products were amplified by the primers Ra ($5'$ -TCTCTGGTTAGACCAGATCTG- $3'$) and U5a ($5'$ -GTCTGAGGGATCTCTAGTTAC- $3'$), and late products were amplified with U5b ($5'$ -GGGAGCTCTCTGGCTAACT- $3'$) and gag ($5'$ -GGATTAA CTGCGAATCGTTC- $3'$) primers. The oligonucleotide probe for real-time PCR was as previously reported²⁷.

Viral assays

For RT-PCR, $1\text{--}2 \mu\text{g}$ RNA was reverse transcribed and amplified by PCR using the Nef primers Na ($5'$ -GACAGGGCTTGGAAGG- $3'$) and Nb ($5'$ -TTAGCAGTCTCGAA GTACTC- $3'$) as described previously²⁸. The integration assay was performed on DNazol-extracted total DNA (Invitrogen) using the Alu primer SB704 ($5'$ -TGCTGGGATTACAG GCGTGAG- $3'$) and primer Rc for the first round of PCR (25 cycles). Nested PCR was performed under the same conditions using primers M667 ($5'$ -GGCTAACTAGGGAA CCCACTG- $3'$) and AA55 ($5'$ -CTGCTAGAGATTTCCACACTGAC- $3'$). For virus production, viral p24 (capsid) was measured by enzyme-linked immunosorbent assay according to the manufacturer's protocol (Beckman-Coulter). Reverse transcription activity was measured as previously reported²⁸.

PKR assay

We electrophoresed $20 \mu\text{g}$ of whole-cell lysates in triple detergent lysis buffer on a 10% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane (Amersham Hybond C+). The membrane was probed with a phospho-Thr 446 PKR-specific antibody or a PKR-specific antibody (Upstate Biotechnology).

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Competing interests statement

The authors declare that they have no competing financial interests.

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E3 ubiquitin ligase that recognizes sugar chains

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N-glycosylation of proteins in the endoplasmic reticulum (ER) has a central role in protein quality control^{1–3}. Here we report that N-glycan serves as a signal for degradation by the Skp1–Cullin1–Fbx2–Roc1 (SCF^{Fbx2}) ubiquitin ligase complex. The F-box protein Fbx2 (ref. 4) binds specifically to proteins attached to

N-linked high-mannose oligosaccharides and subsequently contributes to ubiquitination of N-glycosylated proteins. Pre-integrin $\beta 1$ is a target of Fbx2; these two proteins interact in the cytosol after inhibition of the proteasome. In addition, expression of the mutant Fbx2 Δ F, which lacks the F-box domain that is essential for forming the SCF complex, appreciably blocks degradation of typical substrates of the ER-associated degradation pathway^{5,6}. Our results indicate that SCF^{Fbx2} ubiquitinates N-glycosylated proteins that are translocated from the ER to the cytosol by the quality control mechanism.

In an attempt to identify biological functions for N-glycans, we screened mouse brain extracts for proteins that bind to various sugar probes. When we used GlcNAc-terminated fetuin⁷ (GTF)-immobilized beads, we found two proteins that were specifically eluted with N,N'-diacetylchitobiose (chitobiose) but not with EDTA/EGTA, unlike C-type lectins (ref. 8 and Fig. 1a). Protein sequencing analysis identified them to be Skp1 and Fbx2 (also known as NFB42 for neural F-box relative molecular mass (M_r) 42,000 (42K))⁴. We examined the interaction of Skp1 and Fbx2 with GTF. Fbx2, but not Skp1, interacted with GTF, which indicates that Fbx2 mediates the interaction between Skp1 and GTF. The GTF-binding domain of Fbx2 was mapped to the whole carboxy-terminal domain (residues 95–296; Fig. 1b and Supplementary Information Fig. 1). Fbx2, as well as other F-box proteins, formed a complex with Skp1, Cul1 and Roc1 (Fig. 1c and Supplementary Information Fig. 2).

We devised an *in vitro* ubiquitination assay of GTF by using the recombinant SCF^{Fbx2} complex (ref. 9 and Fig. 1c). Ubiquitination of GTF was detected in a time-dependent manner by immunoblotting with an antibody against fetuin, as judged by the shift in GTF migration to higher molecular mass positions with ubiquitin or GST-tagged ubiquitin (Fig. 1d, lanes 2–5 and 8–9). By contrast, ubiquitination of GTF could not be detected without SCF^{Fbx2} or with SCF ^{β TrCP1} (lanes 6 and 7). The ubiquitination of GTF was inhibited either by chitobiose (Fig. 1e) or when GTF was deglycosylated with the peptide N-glycanase F (PNGase; Fig. 1f). These results show that SCF^{Fbx2} is an E3 ubiquitin ligase that recognizes target proteins in an N-glycosylation-dependent manner.

We used an overlay assay to examine the ability of Fbx2 to bind endogenous proteins (Fig. 2a). Several proteins were detected by full-length Fbx2 and Δ N-2 probes (see Fig. 1b), mainly in the membrane fractions of mouse brains and neuroblastoma cells. The principal protein of M_r 120K (p120) was detected in Neuro2a cells. In the adult mouse brain, we detected a broad protein band at 75K–80K (p75) instead of p120. These interactions were specifically inhibited by chitobiose, but not by other monosaccharides or disaccharides tested (Fig. 2a and data not shown). Removing N-glycans by PNGase completely inhibited Fbx2 binding. In addition, treatment with endo- β -N-acetylglucosaminidase H (endo H), which cleaves the high-mannose type of oligosaccharides, also interfered with the binding. To test whether the interaction of Fbx2 with glycoproteins also occurs *in vivo*, we analysed the proteins that co-immunoprecipitated with Δ N-2 by lectin blotting (Fig. 2b). Several proteins including p120 were detected with Con A, a lectin that binds to high-mannose oligosaccharides, but not with WGA, which binds specifically to terminal GlcNAc or sialic acids. These proteins were not bound to β TrCP1.

We characterized the binding specificity of Fbx2 by pull-down assays using different N-glycans on fetuin and ribonuclease B that had been treated with various glycosidases (Fig. 2c). Fbx2 could bind to mannose-terminated fetuin (MTF) more efficiently than to asialofetuin and DGF, but not to DGF or intact fetuin. Moreover, Fbx2 bound to intact ribonuclease, but its binding was reduced when ribonuclease was treated with α -mannosidase and endo H. We examined the efficiency of eluting Δ N-2 bound to ribonuclease with oligosaccharides and found that Man₉GlcNAc₂ eluted Δ N-2 at much lower concentrations than did chitobiose (Fig. 2d). These

data indicate that N-glycans that contain a diacetylchitobiose structure with mannose residues are required for efficient Fbx2 binding and that further modification of mannose residues by other sugars reduces the Fbx2 binding.

We next investigated whether Fbx2 is involved in proteolysis *in vivo*. Full-length Fbx2 but not Δ N-2 was associated with ubiquitinated proteins in Neuro2a cells under inhibition of proteasome activity, but this association was decreased when chitobiose was included during immunoprecipitation (Supplementary Information Fig. 2), which suggests that the formation of SCF^{Fbx2} is necessary for N-glycoprotein ubiquitination.

We examined the amounts of Fbx2 substrates bound to each construct by overlay assay (Fig. 3a). Despite the presence of a larger amount of substrates in the membrane fraction, only the cytoplasmic substrates were co-immunoprecipitated with Δ N-2. Full-length Fbx2 did not seem to interact with endogenous substrates,

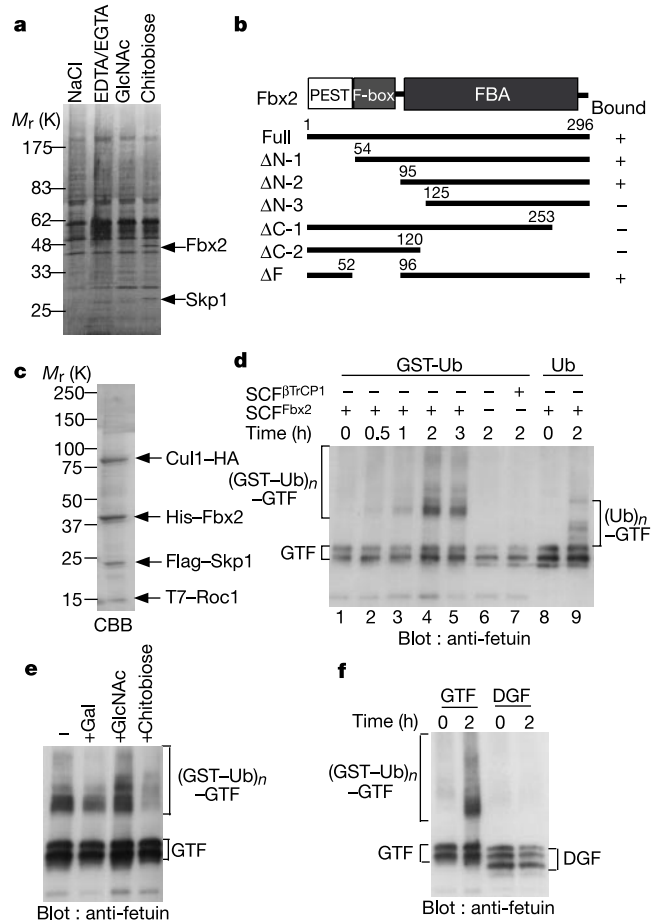


Figure 1 The SCF^{Fbx2} complex ubiquitinates N-glycosylated substrate *in vitro*. **a**, Identification of Fbx2 and Skp1 as GlcNAc-binding proteins. GlcNAc-terminated fetuin (GTF)-bound proteins were eluted with the indicated compounds. The following amino acid sequences were obtained: ⁸³ELVDGAPL and ¹⁸²AQVIDLQAEXY for Fbx2; and ⁸¹RTDDIP and ¹³⁸TFNIK for Skp1. **b**, Interaction between GTF and various mutants of Fbx2. FBA, F-box-associated domain¹⁹. The ability of each Fbx2 deletion mutant to bind GTF is summarized (+ or –; see Supplementary Information Fig. 1 for details). **c**, Electrophoretic pattern of the recombinant SCF^{Fbx2} complex produced by the baculovirus system. **d**, *In vitro* ubiquitination of GTF by the SCF^{Fbx2} ubiquitin ligase system. **e**, Effect of saccharides on *in vitro* ubiquitination of GTF. *In vitro* reactions were done in the presence of 0.1 M galactose (Gal), 0.1 M GlcNAc or 0.1 M chitobiose for 2 h. **f**, N-linked oligosaccharides are required for ubiquitination. *In vitro* ubiquitination was carried out with GTF and deglycosylated fetuin (DGF).

probably owing to proteasomal degradation of substrates. Indeed, the interaction became detectable in the cytoplasm after treatment with MG132. We next purified p120 and determined its amino acid sequence. The peptide-sequence Leu-Pro-Asp-Gly-Val-Thr-Ile-Asn-Tyr-Lys was identical to part of mouse integrin $\beta 1$ (residues 389–398). Previous studies have shown that integrin $\beta 1$ proteins with different oligosaccharide chains run as two separate bands on SDS polyacrylamide gel electrophoresis (SDS–PAGE)^{10,11}. We found that ΔN -2 was co-immunoprecipitated with the precursor (pre-integrin $\beta 1$) but not with the mature form (Fig. 3b). Fbx2 failed to interact with deglycosylated integrin $\beta 1$ in tunicamycin-treated cells but did bind to pre-integrin $\beta 1$, which indicates that Fbx2 interacts specifically with pre-integrin $\beta 1$ that contains high-mannose oligosaccharides (Fig. 3c).

Because ubiquitination occurs in the cytosolic compartment, we determined the site of interaction of Fbx2 with pre-integrin $\beta 1$ by fractionating the Neuro2a cells before immunoprecipitation. Whereas the cytosol fraction contained almost undetectable amounts of pre-integrin $\beta 1$, it did contain pre-integrin $\beta 1$ bound to ΔN -2 (Fig. 3d, lanes 2, 5, 13). Although the interaction between full-length Fbx2 and pre-integrin $\beta 1$ was hardly detected in the cytosol, it was observed on treatment with MG132 (lanes 1, 4). The interaction was also detected in the microsomal fraction, which suggests that pre-integrin $\beta 1$ is bound to Fbx2 on translocation to the cytosol. These results suggest that Fbx2 is responsible for the proteasomal elimination of high-mannose-containing proteins in the cytosol.

Cystic fibrosis transmembrane conductance regulator (CFTR) is degraded rapidly in the cytosol when it fails to properly fold in the ER^{12,13}. This degradation is dependent on the ER-associated

degradation (ERAD) pathway and is enhanced when CFTR has mutations such as $\Delta F508$ (ref. 14). Degradation of misfolded CFTR requires proteasomal activity, and when cells are treated with a proteasome inhibitor CFTR accumulates in intracellular inclusions termed aggresomes¹⁴. We therefore assessed the role of Fbx2 in the degradation of CFTR $\Delta F508$.

Immunoprecipitation of a fusion protein of CFTR $\Delta F508$ and green fluorescent protein (CFTR–GFP) followed by immunoblotting showed that full-length Fbx2, ΔN -2 and ΔF , but not ΔC -1 (see Fig. 1b), could interact with CFTR–GFP; this interaction seemed to be mediated by its glycosylation, because tunicamycin markedly reduced the interaction (Fig. 4a). Because ΔF bound to CFTR–GFP more efficiently than did ΔN -2, we subsequently tested whether the expression of ΔF affects the ERAD pathway. Pulse-chase experiments revealed that whereas CFTR–GFP was degraded rapidly in control cells (17.1% at 2 h), its degradation was significantly suppressed by ΔF overexpression (31.5% at 2 h; Fig. 4b). For another ERAD substrate, the T-cell receptor α -subunit (TCR- α)¹⁵, the decay was more efficiently suppressed by ΔF (at 2 h, vector, 17.7%, ΔF , 70.6%). These results show that Fbx2 is involved in the ERAD pathway *in vivo*. COS7 cells transfected with CFTR–GFP formed a large aggregate adjacent to the nucleus when treated with MG132, as described¹⁴ (Fig. 4c). Such aggresome formation was significantly suppressed when Fbx2 was co-expressed, whereas ΔN -2, ΔC -1, ΔF or full-length β TrCP1 did not cause any change in the formation of aggresomes (Fig. 4d and data not shown). These results suggest that SCF^{Fbx2} is involved in eliminating misfolded glycoproteins that are translocated from the ER to the cytosol.

E3 ubiquitin ligase constitutes a large protein family including

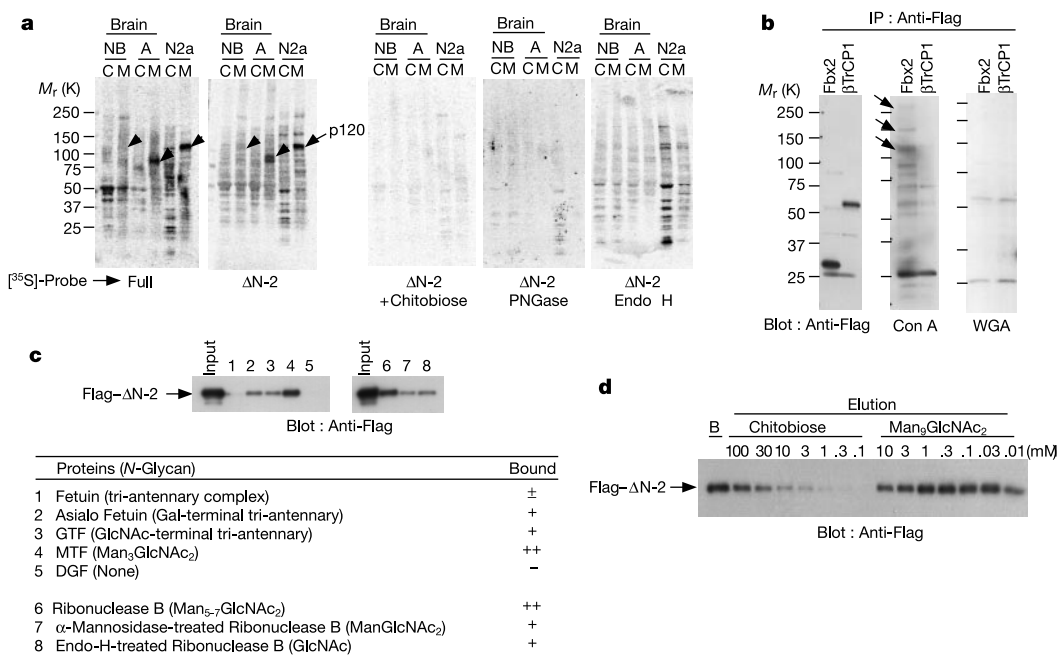


Figure 2 Interaction of Fbx2 with endogenous high-mannose oligosaccharide-containing glycoproteins. **a**, Detection of Fbx2-binding proteins by overlay assay using ³⁵S-labelled Fbx2. Cytoplasmic proteins (C) and membranous fractions (M) in brains of newborn (NB) and adult (A) mice and Neuro2a (N2a) cells were separated by SDS–PAGE and then transferred to membranes. For competition, 0.1 M chitobiose was added with the probe. Each blot was untreated or treated with PNGase or endo H before assay. Arrows indicate p120 and p75. **b**, *In vivo* interaction of Fbx2 with endogenous glycoproteins. Neuro2a cells were transfected with Flag-tagged Fbx2 (ΔN -2) or β TrCP1 lacking its F-box domain.

After immunoprecipitation from whole-cell extracts, the Fbx2-binding proteins were analysed by immunoblotting or lectin-blotting. Arrows denote similar signals to those detected with overlay assay in **a**. **c**, Pull-down analysis of the interaction between Fbx2 and N-glycoproteins. Bound proteins were analysed by immunoblotting (top) and their binding efficiencies were semiquantified (bottom). **d**, Elution of Fbx2 from ribonuclease-B-immobilized beads with oligosaccharides. Ribonuclease-bound ΔN -2 was eluted by boiling with sample buffer (B) or by incubation with chitobiose or Man₃GlcNAc₂.

SCF^{16,17}. F-box proteins are also diverse^{18,19} and are pivotal in selecting target proteins for ubiquitination¹⁶. Accumulating evidence suggests that modification of target proteins is a prerequisite for the recognition by certain SCF-type E3 ubiquitin ligases and subsequent destruction by the 26S proteasome^{16–18}. These include phosphorylation and proline hydroxylation^{20,21}. We have shown here that *N*-glycosylation also acts as a targeting signal to eliminate intracellular glycoproteins by Fbx2-dependent ubiquitination and proteasomal degradation. Fbx2 recognizes high mannose on the substrates. Because high-mannose oligosaccharides are found in the

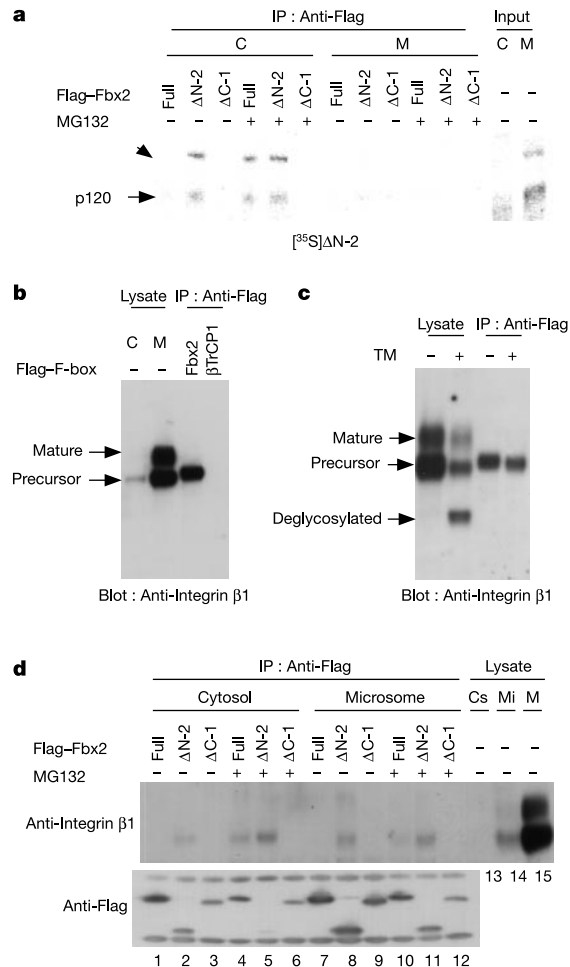


Figure 3 SCF^{Fbx2} promotes proteasomal degradation of cytosolic glycoproteins. **a**, *In vivo* detection of cytoplasmic Fbx2-binding proteins. Neuro2a cells transfected with Flag-tagged Fbx2 were untreated (–) or treated with MG132 (+). After fractionation, Fbx2 was immunoprecipitated from the cytoplasmic (C) and membranous (M) fractions. Lysate (Input) and immunoprecipitates were analysed by overlay assay. Arrows denote similar signals to those observed in Fig. 2a. **b**, Identification of p120 as integrin β 1. Neuro2a cells were transfected with or without Flag-tagged Fbx2 (Δ N-2) or β TrCP1 lacking its F-box domain. Each F-box protein was immunoprecipitated from whole-cell extracts and blotted with integrin β 1 antibody. Cytoplasmic and membranous lysates were loaded to compare the sizes of integrin β 1 proteins. **c**, *In vivo* interaction of Fbx2 with integrin β 1 containing high-mannose oligosaccharides. Neuro2a cells transfected with Flag-tagged Δ N-2 were untreated (–) or treated with tunicamycin (+; TM). After immunoprecipitation of Δ N-2 from whole cell extracts, integrin β 1 proteins were analysed by immunoblotting. **d**, Detection of the Fbx2-integrin β 1 complex in the cytosol. Cytosolic (Cs) and microsomal (Mi) fractions were prepared from cells transfected with or without Fbx2 constructs. The amount of integrin β 1 associated with Fbx2 was determined by immunoblotting.

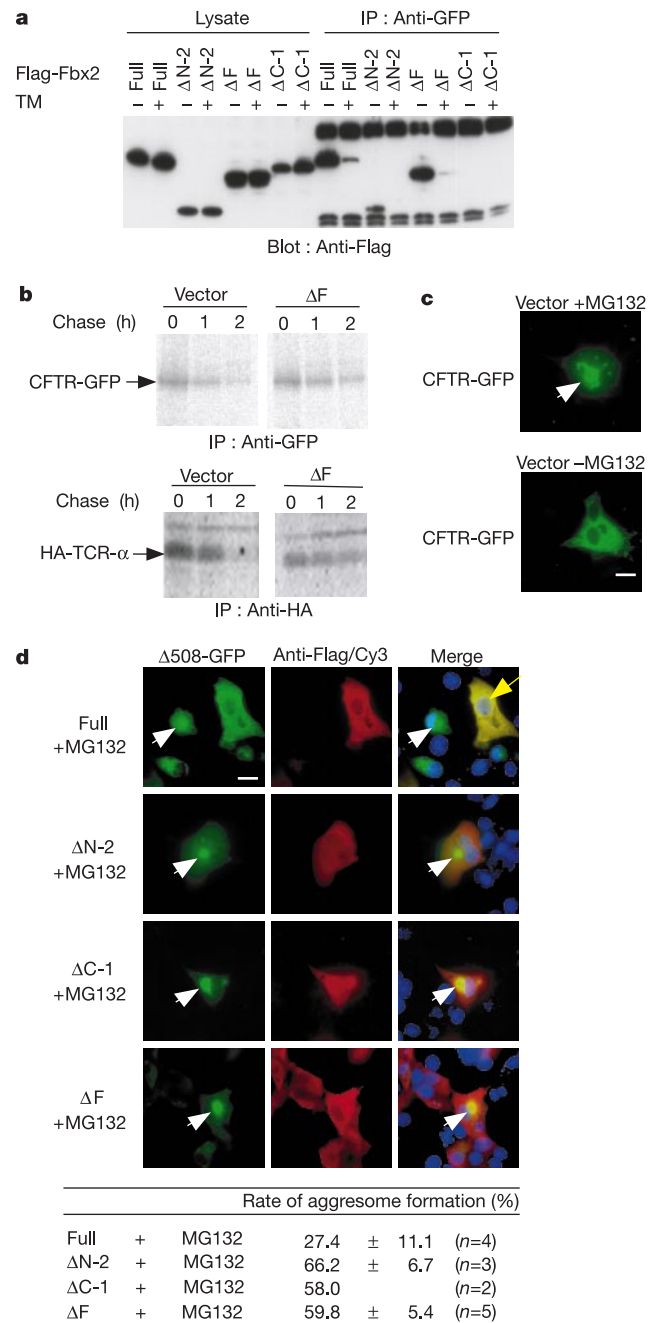


Figure 4 Fbx2 functions in the ERAD pathway. **a**, Interaction between CFTR-GFP and Fbx2. 293T cells transfected with CFTR-GFP and various Fbx2 constructs were untreated (–) or treated with tunicamycin (+; TM). After immunoprecipitation of CFTR-GFP from whole-cell extracts, bound Fbx2 was analysed using an antibody against Flag. **b**, Effect of Δ F on the stability of CFTR and TCR- α . ³⁵S-pulse-labelled (30 min) CFTR-GFP and HA-TCR- α were chased at indicated periods in the absence or presence of Δ F, as described²⁴. **c**, Formation of CFTR-GFP aggresomes by treatment with a proteasome inhibitor. COS-7 cells were transiently transfected with CFTR-GFP, and after 48 h later were either untreated (–) or treated with 20 μ M MG132 (+) in the presence of 10 μ g ml^{–1} cycloheximide for 24 h. Treatment of cells with MG132 resulted in the formation of aggregates of misfolded proteins termed aggresomes (white arrowheads). Scale bar, 10 μ m. **d**, Suppression of aggresome formation by Fbx2. COS-7 cells were transfected with CFTR-GFP and various Flag-tagged Fbx2 constructs. Note the lack of aggresomes (yellow arrowhead) in cells expressing full-length Fbx2. Scale bar, 10 μ m. Rates of aggresome formation in the presence of various Fbx2 constructs are shown. *n*, number of experiments. About 50–100 cells were counted in each experiment. Data are means ± s.e.m.

ER, it is possible that SCF^{Fbx2} is important in quality control coupled to the ER. Indeed, we identified pre-integrin β 1 as a target of Fbx2. Integrin β 1 is expressed in excess over integrin α -chains^{10,22}, and the excess of non-complexed β 1 is either degraded immediately, probably by the ERAD pathway, or remains in the ER by means of calnexin so that it can associate with freshly synthesized α -chains^{10,23}. We have also shown that Fbx2 functions in the ERAD pathway *in vivo* using typical ERAD substrates (Fig. 4b). Thus, we propose that SCF^{Fbx2} is an E3 ubiquitin ligase that is specific for N-linked glycoproteins in the ERAD pathway. It is noteworthy that Fbx2 is expressed mainly in neuronal cells in the adult brain (ref. 4 and our own unpublished data). Accordingly, it is likely that the SCF^{Fbx2}-mediated ERAD pathway contributes to the rapid elimination of glycoproteins that are present in neurons. Further studies should be designed to identify the physiological substrates for SCF^{Fbx2} in neuronal cells and their carbohydrate structure, which may help to understand the *in vivo* function of Fbx2 in the brain. □

Methods

Isolation and identification of GlcNAc-binding proteins

GlcNAc-terminated fetuin (GTF) was prepared by incubating asialofetuin type II (Sigma) with β -galactosidase (*Streptococcus* 6646K, Seikagaku-kogyo) and passed through RCA120 lectin agarose. The brains of 8-week-old ICR mice were homogenized in 10 volumes of TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 5 mM CaCl₂ and protease inhibitor cocktail. After centrifugation of the homogenate at 30,000g for 30 min, the supernatant was incubated with GTF-immobilized beads (Affi-gel 15, Bio-Rad) for 18 h. We washed the beads with TBS containing 0.1% Triton X-100 (TBS-T). The adsorbed proteins were eluted successively by 0.5 M NaCl, 20 mM EDTA/EGTA, and 0.2 M GlcNAc or 0.1 M chitobiose in TBS-T. Eluted proteins were separated by SDS-PAGE, excised and digested with lysendopeptidase, and their sequences were determined by a protein sequencer.

Transfection, immunoprecipitation and immunoblotting

We transfected the 293T, Neuro2a and COS-7 cell lines with various plasmids by lipofection (Lipofectamine Plus, Gibco-BRL). To inhibit proteasome activity, cells were treated with 50 μ M of MG132 (Peptide Institute) for 4 h unless indicated otherwise. To inhibit biosynthesis of N-glycans, cells were treated with 5 μ g ml⁻¹ of tunicamycin for 24 h. Whole-cell extracts were prepared in TBS containing 0.5% Nonidet P-40 (NP-40) and after freeze-thawing were centrifuged at 24,000g for 20 min to produce the cytoplasmic fraction (supernatant) and the membranous fraction (pellet), which was solubilized with 1% Triton X-100. Cytosol and microsome fractions were obtained by centrifugation of the cytoplasmic fraction at 100,000g for 60 min. The microsomal proteins were solubilized with Triton X-100. For immunoprecipitation, we used mouse monoclonal antibodies against Flag (M2; Sigma), Myc (9E10; Santa Cruz), haemagglutinin A (HA.11; Babc) and GFP (clones 7.1 and 13.1; Roche). For immunoblotting, we used mouse monoclonal antibodies against Flag, HA and V5 (Invitrogen), rat monoclonal antibodies against integrin β 1 (Mab1997; Chemicon) and rabbit polyclonal antibodies against Myc (A-14; Santa Cruz) and fetuin (Chemicon).

In vitro ubiquitination assay

SCF^{Fbx2} complex was produced in insect cells by infection with baculovirus encoding His-Fbx2, Flag-Skp1, Cul1-HA and T7-Roc1, and affinity-purified by a HiTrap HP column (Amersham Pharmacia). Other recombinant proteins used for *in vitro* ubiquitination assay have been described⁹. Deglycosylated fetuin (DGF) was prepared by incubating asialofetuin with *Flavobacterium meningosepticum* recombinant PNGase F (Roche). Each 1 μ g of fetuin, GTF or DGF was incubated in 50 μ l of the reaction mixture containing ATP-regenerating system⁹, 0.5 μ g of E1, 1 μ g of Ubc4 (E2), 2 μ g of SCF^{Fbx2} and 6 μ g of bovine ubiquitin (Sigma) or 6.5 μ g of recombinant GST-ubiquitin at 30 °C.

Overlay assay

The cytoplasm and membranous fractions from mouse and cultured cells were separated by SDS-PAGE and blotted onto a membrane (immobilon, Millipore). To prepare ³⁵S-labelled Fbx2 probes, Fbx2-Metx3 (full and Δ N-2) plasmids expressing amino-terminally Flag-tagged full-length Fbx2 and Δ N-2 with an additional three methionines at the C terminus were prepared by subcloning into pTracer-EF (Invitrogen). We used the TNT Coupled Reticulocyte Lysate System (Promega) for generating ³⁵S-labelled Fbx2 and its derivatives. Membranes were blocked in 5% skimmed milk in TBS and incubated with probes in 1% skimmed milk at 4 °C for 18 h. Membranes were washed with TBS containing 0.05% Tween20 and analysed by autoradiography. For treatments with endo H or PNGase, the blotted membranes were blocked with 3% bovine serum albumin in PBS. Each membrane was treated with 1 unit of recombinant endo H (Seikagaku-kogyo) in citrate/phosphate buffer (pH 5.5) or 10 units of PNGase F in phosphate buffer (pH 8.2) at 37 °C for 18 h. The enzyme-treated membranes were washed with TBS and incubated in 5% skimmed milk, before the overlay.

Pull-down assay

We treated 2 mg of asialofetuin or ribonuclease B with various glycosidases (β -galactosidase, jack bean β -N-acetylhexosaminidase, PNGase F, jack bean α -mannosidase or endo H), and then immobilized it to 0.5 ml of Affi-gel 10 or 15. Whole-cell extracts of Δ N-2-expressing cells (25 μ g) were incubated with 15 μ l of various glycoprotein-immobilized beads, and bound proteins were eluted by boiling with SDS sample buffer or incubation with 15 μ l of various concentrations of chitobiose or Man₉GlcNAc₂ at room temperature for 10 min. The eluates were separated by spin filtration.

Immunofluorescence microscopy

COS-7 cells that had been transfected with CFTR-GFP and cultured on glass-bottomed 35-mm dishes were fixed in 4% paraformaldehyde and permeabilized by 0.5% NP-40 in PBS for 30 min. We then washed the cells with PBS and blocked them with 2% fetal calf serum in PBS for 2 h. Fixed cells were incubated with primary antibody and washed with PBS, and then exposed to a 1,000-fold dilution of Cy3-conjugated secondary antibody. We examined immunofluorescence using an Olympus IX70 microscope equipped with a digital camera (C4732-5 Hamamatsu) operated by QED Camera Plug-in software (QED Imaging).

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Competing interests statement

The authors declare that they have no competing financial interests.

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Distinct molecular mechanism for initiating TRAF6 signalling

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Tumour-necrosis factor (TNF) receptor-associated factor 6 (TRAF6) is the only TRAF family member that participates in signal transduction of both the TNF receptor (TNFR) superfamily and the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily^{1–5}; it is important for adaptive immunity, innate immunity and bone homeostasis. Here we report crystal structures of TRAF6, alone and in complex with TRAF6-binding peptides from CD40 and TRANCE-R (also known as RANK), members of the TNFR superfamily, to gain insight into the mechanism by which TRAF6 mediates several signalling cascades. A 40° difference in the directions of the bound peptides in TRAF6 and TRAF2 shows that there are marked structural differences between receptor recognition by TRAF6 and other TRAFs. The structural determinant of the peptide–TRAF6 interaction reveals a Pro-X-Glu-X-X-(aromatic/acidic residue) TRAF6-binding motif, which is present not only in CD40 and TRANCE-R but also in the three IRAK adapter kinases for IL-1R/TLR signalling. Cell-permeable peptides with the TRAF6-binding motif inhibit TRAF6 signalling, which indicates their potential as therapeutic modulators. Our studies identify a universal mechanism by which TRAF6 regulates several signalling cascades in adaptive immunity, innate immunity and bone homeostasis.

The unique biological function of TRAF6 is largely determined by its TRAF-C domain, which does not interact with peptide motifs that are recognized by TRAF1, -2, -3 or -5 (refs 6–8). To elucidate the molecular basis of TRAF6 specificity, we determined the crystal structures (Table 1 and Fig. 1a) of the TRAF-C domain (residues 346–504) alone and in complex with a peptide from human CD40 (residues 230–238, KQEPQEIDF)⁹ or TRANCE-R (residues 342–349, QMPTEDEY)¹⁰. The CD40 peptide contains a mutation (Asn237Asp) that enhances affinity to TRAF6 (ref. 11). Using isothermal titration calorimetry (ITC), we determined the dissociation constant (K_d) between TRAF6 and the nine-residue CD40 peptide to be 84 μ M, which is essentially the same as the K_d between TRAF6 and the whole intracellular segment of CD40 (Supplementary Information). A low affinity between TRAF6 and monomeric receptors is expected, because TRAF recruitment is

dependent on affinity enhancement by receptor and adapter protein oligomerization^{5,12}.

Unexpectedly, there are marked differences in peptide binding to TRAF6 and to other TRAFs. First, the chain direction of bound TRAF6-binding peptides shows a 40° difference to that of TRAF2-binding peptides (Fig. 1b–d). As a result, side chains of TRAF6-binding peptides interact with surface pockets on TRAF6 that are completely different from those on TRAF2. Second, the TRAF6-binding peptides assume extended β -conformations, rather than the poly-proline II (PPII) helix conformation for the core region of TRAF2-binding peptides (Fig. 1b). The peptides also make more extensive main-chain hydrogen bonds with the TRAF-C domain β 7 strand (residues 234–238 of CD40 and 344–349 of TRANCE-R with residues Pro 468 to Gly 472 of TRAF6), which carries the Pro 468 insertion in its β -bulge (Fig. 1e). Finally, the peptides do not interact with the β 3– β 4 loop in the TRAF6 complexes, owing to a movement of 12 Å in the position of this loop (Fig. 1b).

Similar to the nomenclature used for TRAF2-binding peptides, which bear the consensus (Pro/Ser/Thr/Ala)-X-(Gln/Glu)-Glu (corresponding to P₋₂ to P₁; Fig. 1d), we denote residues Glu 235 of CD40 and Glu 346 of TRANCE-R as the P₀ position of TRAF6-binding peptides because they occupy a similar, but not identical, location to the P₀ residue (Gln/Glu) in the TRAF2-binding motif (corresponding C α distance 2.3 Å). The P₀ position is near the point of intersection between the two classes of peptide. For the peptide residues in contact with TRAF6 (P₋₄ to P₃), analyses of surface area burial and specific side-chain interactions suggest that the P₋₂, P₀ and P₃ residues contribute most to the structural interaction (Fig. 1g).

The different surface pocket for the P₋₂ residue seems to be an important factor in determining the different mode of peptide binding by TRAF6 relative to TRAF2. Ser 467 and Cys 469, which form the P₋₂ pocket in TRAF2, are replaced in TRAF6 by Phe 471 and Tyr 473, which form an alternative pocket for the P₋₂ proline residue that is located about 3 Å from that in TRAF2 (Fig. 1c–e). The carboxylate of the P₀ glutamic acid residue is recognized by hydrogen bonding with main-chain amide nitrogen atoms of Leu 457 and Ala 458, and the aliphatic portion of the side chain shows a close fit with the TRAF6 surface (Fig. 1c, e). This mode of interaction is in contrast to the recognition of the P₀ glutamic acid/glutamine by the hydroxyls of three serine residues (453–455) in TRAF2. In addition, the carboxylate of the P₀ residue may form a favourable charge–charge interaction with the side chain of Lys 469, although the interaction is not within hydrogen-bonding distance. The P₃ residue, Phe 238 of CD40 or Tyr 349 of TRANCE-R, is adjacent to several aromatic and basic residues of TRAF6 (Fig. 1e). An amino–aromatic interaction is observed between Tyr 349 of TRANCE-R and Arg 392 of TRAF6. Structurally, a similar interaction should be possible for either Phe 238 of CD40 or an acidic residue, which is present in mouse CD40 (Fig. 1g). The observed difference in the side-chain conformation of the P₃ residue in CD40 is probably caused by crystal packing (Fig. 1f).

Structure-based sequence alignment of TRAF6-binding sites in human and mouse CD40 and TRANCE-R led to the definition of a TRAF6-binding motif for P₋₂ to P₃ of Pro-X-Glu-X-X-(Ar/Ac), where Ar is an aromatic and Ac an acidic residue (Fig. 1g). An *in vitro* binding assay between glutathione S-transferase (GST)-fused full-length cytoplasmic domain of CD40 (GST-CD40ct) and TRAF6 (residues 333–508) confirmed the structural prediction that the P₋₂, P₀ and P₃ residues are the most crucial for TRAF6 interaction (Fig. 1g). Similarly, in a cellular assay of the ability of CD40 to activate the NF- κ B transcription factor¹¹, mutations of the same three residues significantly decreased NF- κ B reporter activity as compared with wild-type CD40 (Fig. 2a). The P₁ and P₂ positions may have a preference for acidic residues owing to their complementarity to the basic TRAF6 surface (Fig. 1c), which is formed in particular by the side chains of Arg 392 and Lys 469 at this region.

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Table 1 Crystallographic statistics

Crystal	Native	hCD40 complex	hTRANCE-R complex
TRAF6	Residues 346–504	Residues 346–504	Residues 346–504
Peptide	None	230-KQEPQEIDF	342-QMPTEDEY
Space group	$P2_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions	$a = 32.2 \text{ \AA}$, $b = 55.6 \text{ \AA}$, $c = 47.7 \text{ \AA}$, $\beta = 101.0^\circ$	$a = 39.9 \text{ \AA}$, $b = 43.8 \text{ \AA}$, $c = 101.4 \text{ \AA}$	$a = 38.0 \text{ \AA}$, $b = 45.0 \text{ \AA}$, $c = 106.5 \text{ \AA}$
Diffraction data			
Resolution	40–2.4 \AA	40–1.8 \AA	40–2.0 \AA
R_{sym} (last shell)	9.2% (24.1%)	5.6% (22.1%)	5.5% (13.9%)
Completeness (last shell)	92.8% (90.4%)	99.1% (93.0%)	96.0% (86.9%)
Refinement			
Resolution	20–2.4 \AA	20–1.8 \AA	20–2.0 \AA
Sigma cut-off	2.0	2.0	2.0
Number of protein residues	155	164	161
Number of protein atoms	1,269	1,340	1,331
Number of solvent atoms	45	122	80
Number of reflections used	5,538	14,644	12,396
R (R_{free})	20.4% (27.4%)	20.3% (25.8%)	21.3% (24.2%)
r.m.s. deviation bond length	0.007 \AA	0.014 \AA	0.005 \AA
r.m.s. deviation bond angle	1.4^\circ	1.8^\circ	1.3^\circ

ITC measurements showed that peptides with acidic residues at these positions possess higher affinity to TRAF6 (Supplementary Information). However, mutations of the P_{-2} , P_0 and P_3 residues of the TRANCE-R sequence in the full-length receptor failed to reduce TRANCE-R-induced NF- κ B activation (ref. 13 and Supplementary Information). Examination of the TRANCE-R sequence suggested that there are three potential TRAF6-binding sites (Fig. 1g, h). To evaluate the functional roles of these sites, we generated single,

double and triple mutations at each of the P_0 positions to disrupt one, two or three of the TRAF6-binding sites (Fig. 2b). Neither single nor double mutations resulted in a significant reduction of NF- κ B activation; however, the triple mutation reduced NF- κ B reporter activity to less than 20% of that of wild-type TRANCE-R, which suggests that the three sites are redundantly involved in TRAF6 interaction and signal transduction. The remaining NF- κ B activity induced by the triple TRANCE-R mutant can be attributed to the presence of TRAF2-binding sites in full-length TRANCE-R. It

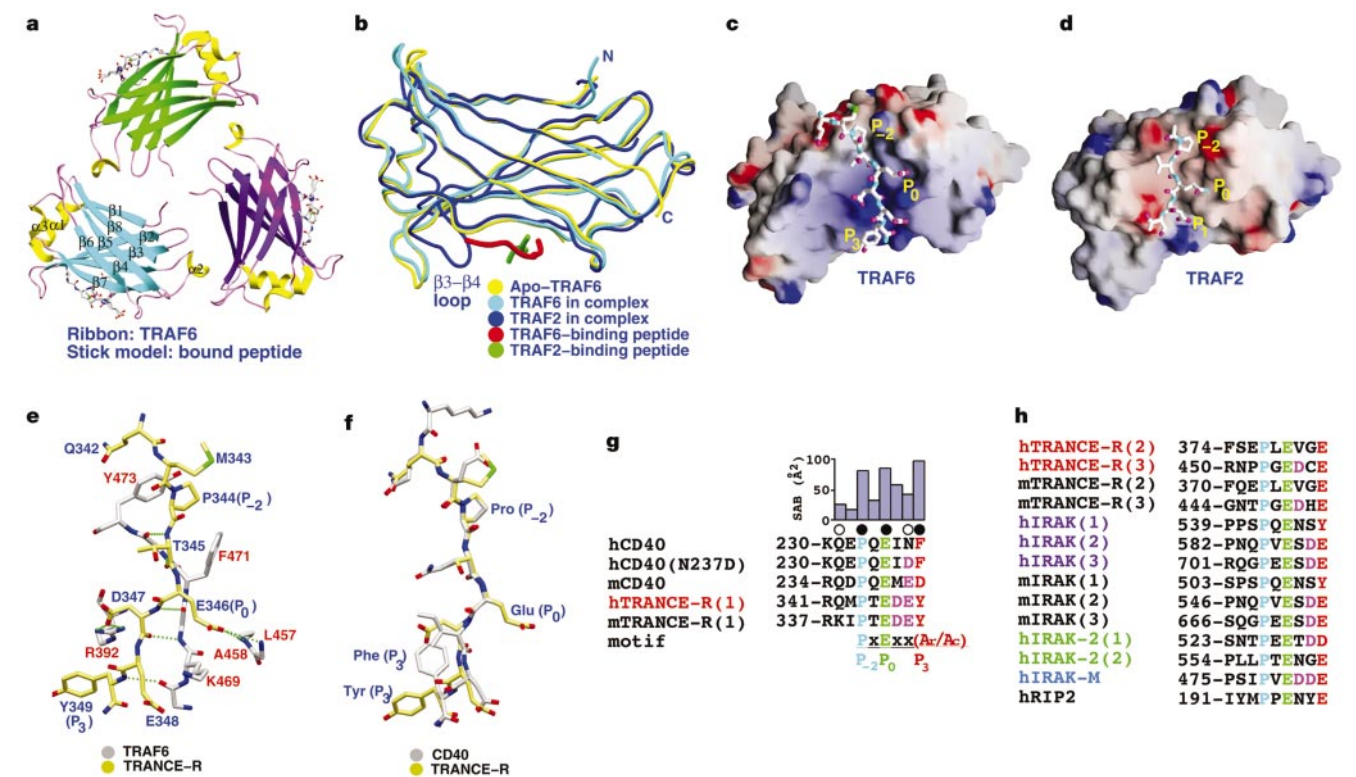


Figure 1 TRAF6 structures. **a**, Ribbon diagram of the TRAF domain of TRAF6 in complex with TRANCE-R, shown as a trimeric model by applying the putative three-fold symmetry observed in TRAF2 structures⁶. **b**, Worm C α traces of superimposed TRAF6 and TRAF2 structures. **c**, Surface representation of TRAF6, coloured on the basis of electrostatic potential ($-10k_bT/e$ to $+10k_bT/e$, where k_b , T and e are the Boltzmann constant, temperature and the electron charge, respectively), and the bound TRANCE-R peptide. **d**, Surface representation of TRAF2, shown with the bound core CD40 peptide. **e**, Interaction between TRAF6 and the TRANCE-R peptide. Main-chain hydrogen bonds

between the TRANCE-R peptide and the $\beta 7$ strand of TRAF6 are shown as dotted lines. Side chains of some of the $\beta 7$ residues are omitted for clarity. **f**, Superposition of the TRANCE-R and the CD40 peptide. **g**, The Pro-X-Glu-X-X-(Ar/Ac) TRAF6-binding motif. The surface area buried (SAB) on TRAF6 interaction for the eight contacting residues (P_{-4} to P_3) is shown. CD40 residues that were mutated to assess their effect on *in vitro* interaction with TRAF6 are indicated on top by a circle (open, does not abolish interaction; filled, abolishes interaction). **h**, Presence of one or many Pro-X-Glu-X-X-(Ar/Ac) motifs in TRANCE-R, IRAK, IRAK-2, IRAK-M and RIP2.

is possible that the presence of several TRAF6-binding sites in TRANCE-R increases the probability of TRAF6 recruitment and explains the dominant role of TRAF6 in TRANCE-R signalling^{2,3}.

The best-characterized TRAF6 signalling pathway for the IL-1R/TLR superfamily involves IRAK, an adapter kinase upstream of TRAF6 (refs 1, 14, 15 and Fig. 1h). On receptor stimulation, IRAK becomes oligomerized and interacts with TRAF6 (ref. 12). Full-length IRAK contains three potential TRAF6-binding sites (Fig. 1h), which suggests that it may directly initiate TRAF6 signalling through the same structural mechanism. To test the functional importance of these sites, we generated a series of single, double and triple mutations of the respective P₀ residues (Fig. 2c). On transfection, wild-type IRAK strongly promoted NF- κ B reporter activity, whereas single, double and triple P₀ mutations attenuated NF- κ B activity relative to the wild type. The degree of attenuation increased with the number of P₀ mutations, with the triple mutation showing NF- κ B activation that was close to the background activation. All of the IRAK mutants showed similar amounts of expression (Supplementary Information). These observations suggest that the three TRAF6-binding sites in IRAK contribute collectively to TRAF6 activation.

The identification of the Pro-X-Glu-X-X-(Ar/Ar) motif prompted us to inspect sequences of other proteins that may directly interact with TRAF6 (Fig. 1h). We found that the IRAK

homologues IRAK-M¹² and IRAK-2 (ref. 16) contain, respectively, one and two potential TRAF6-binding sites. This is in keeping with the implicated role of IRAK-2 and IRAK-M in IL-1 signalling^{12,16} and the role of IRAK-2 in TLR4 signalling^{17,18}. In addition, we found that the kinase RIP2, which can activate NF- κ B and induce cell death¹⁹, also contains a putative TRAF6-binding site.

We showed further that the direct interaction between TRAF6 and the Pro-X-Glu-X-X-(Ar/Ar) motifs in IRAK and IRAK homologues is responsible for initiating TRAF6 signal transduction under endogenous conditions using dominant-negative TRAF6 (T6.DN) with a mutation at Arg 392, Phe 471 or Tyr 473. Whereas wild-type T6.DN could compete with and downregulate endogenous IL-1-induced TRAF6 signalling, the mutant T6.DN proteins, which failed to interact with the Pro-X-Glu-X-X-(Ar/Ar) motif *in vitro* (Supplementary Information), showed significantly reduced inhibition of NF- κ B activation (Fig. 2d). All T6.DN mutants were expressed in similar quantities (Supplementary Information).

We tested whether peptides derived from the TRAF6-interacting motif could inhibit TRAF6-mediated signal transduction. We generated cell-permeable decoy peptides, L-T6DP-1 and L-T6DP-2

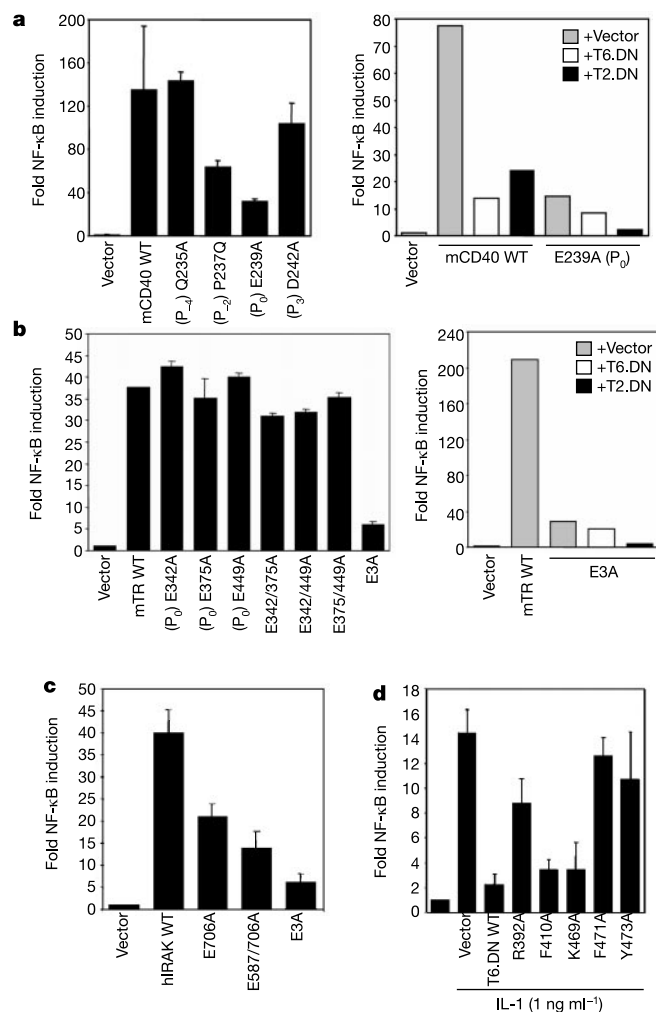


Figure 2 Functional analyses for the interaction of TRAF6 with CD40, TRANCE-R and IRAK. **a**, NF- κ B induction by mouse CD40. **b**, NF- κ B induction by mouse TRANCE-R (mTR). **c**, NF- κ B induction by human IRAK. **d**, Inhibition of endogenous IL-1 signalling by mouse T6.DN (residues are numbered on the basis of human TRAF6).

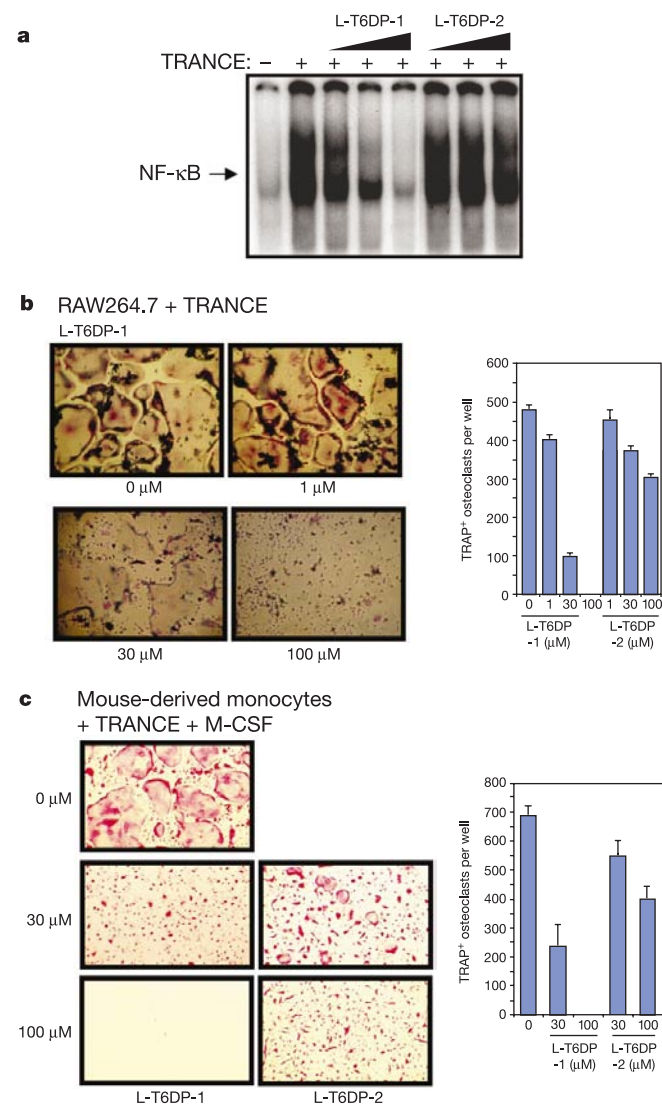


Figure 3 Inhibitory effects of TRAF6 decoy peptides (L-T6DP-1 and L-T6DP-2) in TRANCE-mediated signal transduction and osteoclast differentiation. **a**, Inhibition of TRANCE-mediated NF- κ B activation by TRAF6 decoy peptides, as shown by EMSA. **b**, **c**, Inhibition of TRANCE-mediated osteoclast differentiation in RAW264.7 cells (**b**) and primary monocytes (**c**) by TRAF6 decoy peptides. Cells were stained for TRAP.

2, by fusing a hydrophobic sequence of the Kaposi fibroblast growth factor signal peptide²⁰ with sequences containing TRANCE-R(1) and TRANCE-R(2), respectively (Fig. 1g, h). TRANCE-R(2) does not contain acidic residues at either the P₁ or P₂ position, which may explain why it shows a much weaker affinity for TRAF6 than that of TRANCE-R(1), as measured by ITC (K_d , 770 μ M and 78 μ M, respectively).

We used RAW264.7 cells, which express endogenous TRANCE-R, to test the effects of the decoy peptides, because it is desirable to block TRANCE-R-mediated osteolytic activity²¹ in many human diseases such as osteoporosis and cancer-induced bone lesions. Pre-treatment with L-T6DP-1 inhibited endogenous TRANCE-R-mediated NF- κ B activation on TRANCE stimulation in a dose-dependent manner (Fig. 3a). The effective concentration of L-T6DP-1 is consistent with the affinity between TRANCE-R(1) and TRAF6. The L-T6DP-2 peptide did not produce detectable inhibition of NF- κ B activation, which is consistent with the much weaker affinity of TRANCE-R(2) for TRAF6 and indicates the specific effects of these peptides.

We further tested whether TRANCE-induced osteoclast differentiation can be blocked by TRAF6-binding decoy peptides in a model cell line and in primary cells. After stimulation with TRANCE and TRANCE plus macrophage colony-stimulating factor (M-CSF), respectively, RAW264.7 cells and primary mouse-derived monocytes differentiated into multinucleated, tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts. Co-treatment of RAW264.7 cells and primary monocytes with L-T6DP-1 or L-T6DP-2 caused a dose-dependent decrease of TRAP-positive osteoclasts (Fig. 3b, c) without affecting cell viability (Supplementary Information). These results show that peptides containing the TRAF6-binding motif can act as decoys to inhibit TRAF6 signalling and its associated biological functions.

In summary, our structural and functional study identifies a universal structural mechanism by which TRAF6 participates in adaptive immunity, innate immunity and bone homeostasis. This molecular understanding provides not only insights into the signalling mechanism, but also tools to modulate TRAF6-mediated biological processes. □

Methods

Protein expression, purification and crystallization

A combination of genetic, biochemical and crystallographic methods was used to identify a construct of TRAF6 that gives well-diffracting crystals. In brief, we screened many TRAF6 deletions and found that the construct (residues 333–508) containing part of the coiled-coil domain and the whole TRAF-C domain produced soluble protein that existed predominately as trimers in solution. Poorly diffracting crystals could be obtained, but only at very low protein concentrations. Crystal packing revealed by the molecular replacement solution showed that the TRAF6 construct was monomeric in the crystal with presumably a disordered coiled-coil domain. Removal of most of the coiled-coil domain led to a new monomeric TRAF6 construct (residues 346–504), which showed low solubility but produced well-diffracting crystals. Both TRAF6 constructs contain carboxy-terminal histidine tags. They were purified by Ni²⁺-affinity chromatography and gel filtration, and crystallized under 5–25% PEG8000 in 100 mM Tris-HCl (pH 7.5). For co-crystallization, a 10-fold molar excess of TRAF6-binding peptides was included in the crystallization drops.

Isothermal titration calorimetry

Peptides containing putative TRAF6-binding sequences were synthesized chemically with N-terminal acetylation and C-terminal amidation. The TRAF6 (residues 333–508) and peptide samples were dialysed extensively against 50 mM sodium phosphate at pH 7.5 for at least 2 days at 4 °C to ensure buffer equilibration. Accurate concentrations of the TRAF6 and peptide samples after dialysis were determined by quantitative amino acid analysis. We carried out ITC experiments at 20 °C using a microcalorimetry system (MicroCal). Each peptide (1.0–5.0 mM) was titrated into a TRAF6 sample (0.05–0.1 mM) in roughly 20–45 serial injections. Each titration data set was corrected for heat of dilution, obtained by injecting the peptide into the buffer, and analysed by the ORIGIN software²².

Data collection and structure determination

Diffraction data were collected at the X4A beamline of NSLS and the A1 beamline of CHESS and processed with the HKL package²³. The structures were determined by molecular replacement calculations in the program Replace²⁴ using a TRAF domain structure of TRAF2 (ref. 8) as a search model after removal of non-conserved side chains.

We used the simulated annealing protocol in CNS²⁵ for structure refinement and the program O²⁶ for model building. Ribbon and stick models were created using Setor²⁷ and molecular surface representations were calculated and presented by Grasp²⁸.

In vitro interaction assay

The interaction of TRAF6 (residues 333–508) with the GST-fused intracellular domain of human CD40 (GST-CD40ct, residues 216–273) was carried out by native PAGE and size-exclusion chromatography. We used the PhastSystem (Pharmacia) and 8–25% gradient polyacrylamide gels for native PAGE, and Superdex 200 HR 10/30 (Pharmacia) for size-exclusion chromatography. GST-CD40ct formed oligomers in solution that could interact with the trimeric TRAF6 construct.

Transfection and NF- κ B reporter assays

Mouse CD40 was cloned by polymerase chain reaction with reverse transcription from whole spleen messenger RNA and inserted into the pFlag-CMV1 vector (Sigma). Construction of Flag-tagged mouse TRANCE-R, and mouse dominant-negative TRAF6 (T6.DN, residues 289–530) and TRAF2 (T2.DN, residues 241–501) have been described¹³. Flag-tagged human IRAK was provided by Z. Cao (Tularik). We transfected 293T HEK cells in six-well plates with wild-type or mutant CD40 (100 ng), TRANCE-R (50 ng), IRAK (100 ng), T6.DN (800 ng) or T2.DN (800 ng) along with an NF- κ B-luciferase reporter plasmid (75 ng) and a β -galactosidase plasmid (25 ng) to control for transfection efficiency. Transfection amounts were kept constant by adding empty pFlag-CMV1 vector. Cells were collected 24–30 h after transfection, and reporter activity was assayed as described¹³. Where indicated, cells were treated with 1 ng ml⁻¹ recombinant human IL-1 (R & D) 6 h before collection. Relative luciferase activity was normalized for β -galactosidase activity. Representative results of at least three independent transfections were obtained (error bars are shown for duplicates or triplicates at each transfection).

Decoy peptides

TRAF6-binding sequences from mouse TRANCE-R fused with the hydrophobic sequence from Kaposi fibroblast growth factor signal sequence (L-T6DP-1, AAVALLPAVLLALLAP-RKIPTDEYTDPRSPQST; L-T6DP-2, AAVALLPAVLLALLAP-IPPFQEPLEVGEVD; leader sequence is shown in italic and the TRAF6-binding motif is underlined) were chemically synthesized and purified by high performance liquid chromatography. We confirmed the molecular mass of each peptide by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Electrophoretic mobility shift assays (EMSA)

We plated RAW264.7 cells into six-well plates, incubated them with L-T6DP-1 or L-T6DP-2 (30 μ M, 100 μ M and 300 μ M) for 5 h, and treated them with 10 nM TRANCE for 15 min. Nuclear extracts were prepared as described²⁹. Equivalent amounts of nuclear protein were used in an EMSA reaction with ³²P-labelled NF- κ B oligonucleotide from the HIV-LTR as described²⁹.

In vitro osteoclast differentiation

Primary bone marrow monocytes and RAW264.7 cells were cultured in 48-well dishes at a density of 1×10^5 cells per well and 2×10^3 cells per well, respectively. They were treated with 50 ng ml⁻¹ TRANCE and 10 ng ml⁻¹ M-CSF (for bone marrow monocytes) at the beginning of the culture and during a medium change on day 3 (for bone marrow monocytes). We assessed osteoclast formation by counting the total number of multinucleated (>3 nuclei), TRAP-positive cells per well on day 7 for primary monocytes and on day 5 for RAW264.7 cells³⁰.

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Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to H.W. (e-mail: haowu@med.cornell.edu). The atomic coordinates have been deposited in the Protein Data Bank under accession numbers 1LB4 (native TRAF6), 1LB5 (TRAF6-TRANCE-R complex) and 1LB6 (TRAF6-CD40 complex).

erratum

Regulation of *Arabidopsis* cryptochrome 2 by blue-light-dependent phosphorylation

Dror Shalitin, Hongyun Yang, Todd C. Mockler, Maskit Maymon, Hongwei Guo, Garry C. Whitelam & Chentao Lin

Nature **417**, 763–767 (2002).

In Fig. 4a the third and fourth lanes should have been labelled R₆₀ and B₆₀ (not R₃₀ and B₃₀). □

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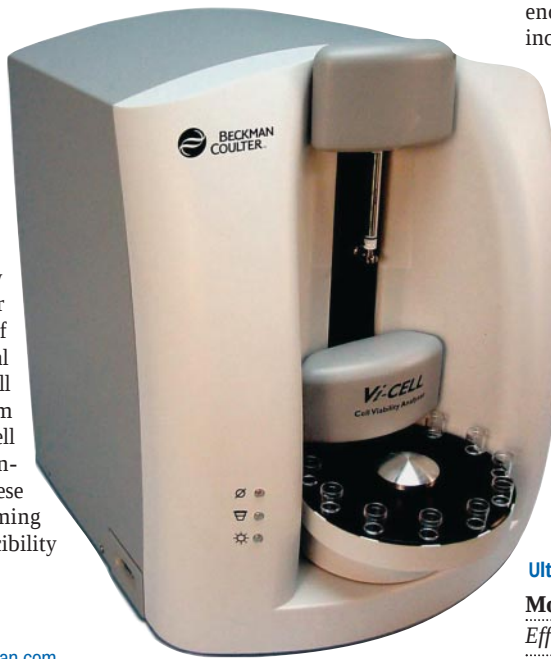
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These notes are compiled in the Nature office from information provided by the manufacturers.

DRUG DISCOVERY



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Screening for drug discovery: The leading question

technology feature

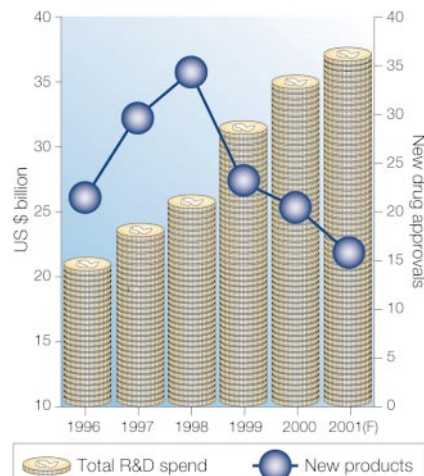
All pharmaceutical researchers know the feeling. Somewhere out there must be that elusive molecule — one that will inhibit this enzyme or activate that receptor in the way they want, and without causing unwanted side-effects. But finding it is another matter. For small-molecule drugs — the mainstay of the pharmaceutical industry — time-consuming and expensive screening is needed to pick out promising candidates from the vast number of natural and synthetic compounds available. Testing large numbers of compounds to see if they produce an appropriate biochemical or cellular effect is usually one of the first steps in the drug-discovery pathway, and ways of making this screening faster, more effective and less expensive are in continual development.

A positive response in a first round of screening in a biochemical assay identifies the primary 'hit' compounds. These molecules then go into more screens to see if they have physicochemical and pharmacological properties that are not too incompatible with making a drug — if it passes this filter, a hit becomes a 'lead'.

Lead compounds then undergo further rounds of chemical refinement and biological screening before finally entering clinical testing. With a good deal of luck, your lead might eventually be approved as a drug 12–15 years after testing began.

But all is not quite as it should be in the drug industry. Estimates vary, but in general analysts agree that each major pharma company needs to launch three or four new products a year in order to sustain the present level of growth. A glance at the chart on the right shows that productivity over the past few years has been well below this level, with the top 20 pharma companies averaging just over one new launch a year.

Increasing the number of leads is thus high on the drug-discovery agenda. Foreseeing the coming deficit, companies implemented a number of strategies in the late 1980s intended to do this. Combinatorial chemistry was used to generate larger libraries of compounds for testing, and high-throughput technology, including increasing miniaturization and automation, was deployed to screen these



R&D spend versus new drug launches by the top 20 pharma companies.

libraries more rapidly. But despite tremendous advances in all aspects of the screening process, chiefly the increased use of automation, these improvements did not bring about the expected rise in productivity, and the industry's drug pipelines still look decidedly thin.

Advocates of high-throughput screening claim that the technique is still in its infancy. "High-throughput screening is not 100,000 tests a day, it's 100,000 tests every day," says

AUTOMATING THE SCREENING PROCESS

Many companies are finding that installing an efficient high-throughput screening facility is as much about introducing new methods of workflow management as it is about getting the technology right. A single screen involves about 80–100 separate activities, such as making sure each reagent is available, producing recombinant protein and plating the compounds. A major pharmaceutical company could be doing 50–100 screens a year, 40–50 of which may be running concurrently. To keep these running efficiently needs collaborative planning and a commitment to an effective supply chain.

"If the plates don't turn up on the Monday morning, your screen just isn't going to get done. This can't be planned in a lab book," says Richard Archer, chief executive of The Automation Partnership, a company based in Royston, UK, which supplies high-throughput screening equipment. But the basics of supply-chain management do not come easily to scientists, says Archer, as they tend to get excited by the one novel assay they're planning, rather than by the prospect of keeping the more conventional assays running.

Another difficulty in implementing high-throughput screening is the bias that has existed when it comes to recruiting 'screeners' as opposed to research-focused PhDs. "You get higher up the tree by being a bright scientist than being an efficient process manager," says Mark Beggs, head of consulting



Tip washing in TAP's Asset screening platform

biotech firm Amgen set up a screening facility at its headquarters in Thousand Oaks, California, it brought in a strong engineering team of technical support staff to keep the equipment working. "It seemed to them to be the obvious thing to do, and the firm was surprised to find that this was fairly unique in the industry," says Archer.

at The Automation Partnership. "The person who discovers the kinase will get further than the one who instigates highly productive screens to identify inhibitors for it."

Despite these hurdles, process management is increasingly seen as being key to successful screening. Equipment manufacturer Amersham Biosciences, based in Piscataway, New Jersey, recently struck a deal with Cimarron Software, Inc, a laboratory workflow management consultancy based in Salt Lake City, Utah. And when

THE AUTOMATION PARTNERSHIP

Richard Archer, chief executive of The Automation Partnership in Royston, UK, a company that makes automated equipment for screening. "You can't say that automated high-throughput screening doesn't work, because nobody is doing it yet."

But all now agree that for screening, quality is more important than quantity. Throughout the industry the emphasis is shifting — from screening the greatest number of compounds as quickly as possible, to making sure that high-quality compounds are going into robust and reproducible assays, and to understanding potential targets better (see "Getting to know the family", below).

Getting organized

The basic workhorse of screening is the microtitre plate, and a number of companies have developed robust, automated screening platforms based on this format. Initially, the plates featured 96 wells, which allowed the same number of different molecules to be screened for a given activity. More recently, the miniaturization of the simpler types of assay has seen the 384-well plate become standard, leaving those with 96 wells to be used in more complicated assays.

Plates with an even higher capacity (1,536 and even 3,456 wells) are used more rarely because the problems associated with handling minute volumes can add significantly to the cost of the assay (see

Portable screening: Amersham's LEADseeker

"How small should you go?", page 457). Amersham Biosciences, an equipment manufacturer based in Piscataway, New Jersey, is one of several companies producing the new generation of robust screening platforms. Its LEADseeker, for example, is designed for decentralized primary screening. It uses imaging technology based on a charge-coupled device that detects fluorescence and luminescence, and allows a whole 96-, 384- or 1,536-well plate to be read at a time.

Amersham sees the LEADseeker as a step on from earlier technologies based on tracking radiolabelled samples, such as scintillation proximity assays. Indeed, a

general feature of the new generation of equipment is that it uses fluorescence-based assays. These have high signal-to-noise ratios, and therefore offer higher-quality data compared with radioactivity-based assays — so much so that in many cases signal detection is so clear there is no need to do replicate wells.

But supplying the equipment is just one part of the equation. Manufacturers also recognize that organizing the high-throughput laboratory's workflow is equally important (see "Automating the screening process", page 453). "Different companies want different things," says Mike Evans, vice-president for bioassays at Amersham. "Some want 'turnkey solutions', whereas others want to mix and match with piecemeal technology."

Making products tailored to an individual user's requirements is also becoming a common theme among providers of software and bioinformatics solutions for screening. Here, the main cry from the industry is for data-handling packages that conform to common standards so that they can be interfaced with existing systems. "In the past, software companies were sometimes guilty of trying to impose their own standards on the industry," says Scott Kahn, a senior vice-president at Accelrys, a software manufacturer in San Diego, California.

Accelrys is one of several companies that favour the development of generally



AMERSHAM BIOSCIENCES

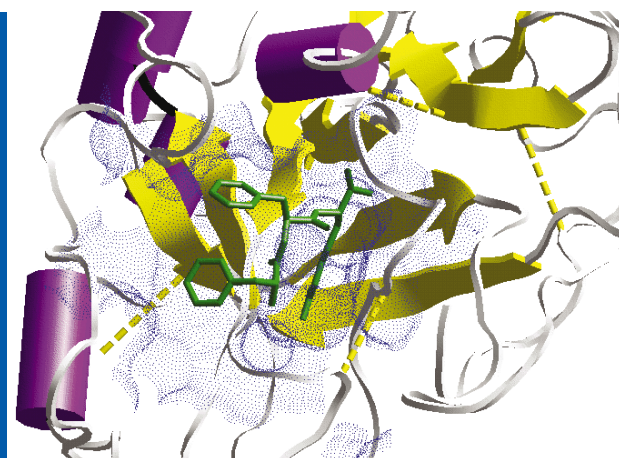
GETTING TO KNOW THE FAMILY

Most companies seek hits against members of the main families of proteins known to be likely drug targets, such as G-protein-coupled receptors, kinases and proteases. Not surprisingly, achieving selectivity for just one member of such a family can be a considerable challenge.

"Just about anybody can get a hit against a kinase," says Richard Scott, head of chemoinformatics at De Novo Pharmaceuticals, a company based in Cambridge, UK, that uses virtual screening to discover drug leads. "It's easy to get a hit, but not nearly so easy to get a selective hit."

As a result, many companies are striving to get to know their target protein families better. This approach has always been central to the philosophy of Vertex Pharmaceuticals, a drug-discovery firm in Cambridge, Massachusetts, but Mark Namchuk, head of high-throughput screening at the company, says that this is now a trend throughout the industry. At Vertex, early discovery is focused on whole protein families rather than individual target proteins.

Emphasizing just how central an understanding of the biology of the target family is to the usefulness of the screening data, the high-throughput screening facility at Vertex is run as a division of the enzymology group, which refers to screening as "high-throughput enzymology", and to screening campaigns as "experiments". As well as giving valuable insights into selectivity, "this approach allows you to view today's data as foundations for



DE NOVO PHARMACEUTICALS

future projects", Namchuk says.

Although not yet a replacement for bioassaying the activity of your molecule on the proteins themselves, virtual screening of similar targets can help in identifying problems that might crop up further down the pipeline. "Virtual screening can give you a heads-up to other potential interactions," says Scott Kahn, a senior vice-president at software manufacturer Accelrys in San Diego, California.

A.S.

recognized external, non-proprietary standards. The company uses Microsoft standards, as does its main competitor, Spotfire in Somerville, Massachusetts. This offers the end-user additional benefits. “The fact that both companies are developing to a common standard means that although they’re competing head-to-head, users can integrate their products however they wish,” says Kahn.

The shape of things to come

Libraries of small-molecule compounds are the raw material that goes into the primary screens. Although there is general agreement about how assay platforms should be developing, there seems to be little consensus about the shape of the ideal compound library. Opinions vary on how big a library should be, and how companies should design, store and handle its contents.

One idea that is exciting interest is to profile and filter compounds for drug-like properties such as solubility and lipophilicity before they ever get into the library. This should give medicinal chemists an easier time by ensuring that lead compounds need less refinement to turn them into drugs.

Companies such as Argenta Discovery, a medicinal chemistry design and screening company based in Harlow, UK, are now screening compounds for a range of drug-like behaviours before they enter

the company’s libraries. Chris Newton, chief scientific officer at Argenta, describes the profiling as “multi-parametric optimization”.

Early whittling away of compounds with undesirable properties can also be done by computer, and *in silico* screening for ‘drug-likeness’ is a central component of the ‘virtual-screening’ strategies of companies such as Argenta, De Novo Pharmaceuticals in Cambridge, UK, and Vertex Pharmaceuticals in Cambridge, Massachusetts. “We’re trying to encode the common sense of medicinal chemists into the computer,” says Mark Namchuk, head of high-throughput screening at Vertex. The company uses a proprietary program called REOS (rapid elimination of swill) to eliminate non-drug-like molecules before compounds make it through to the primary screen.

It is too early to judge the success of virtual-screening programmes, but two independent teams of researchers, from Merck laboratories in Rahway, New Jersey, and from Brian Shoichet’s group at Northwestern University, have shown structure-based computational docking used as a filter can hugely enrich the hit rate compared with random screening.

Compounds on display

One area of chemical screening where the drive towards automation has been somewhat weak is compound handling.



Namchuk:
screens are
experiments.

The preparation of microtitre plates — placing the various compounds into their appropriate wells ready for screening — is still relatively slow.

Graffinity Pharmaceuticals, a drug-discovery company based in Heidelberg, Germany, has come up with an alternative strategy. It

sprays 10,000 compounds as spots onto a ‘chip’, and their affinity for a target protein can be read simultaneously by an imager based on the surface plasmon resonance method developed by equipment manufacturer Biacore in Uppsala, Sweden (see “Fragmenting the problem”, page 459).

Graffinity’s early microarrays were made up of binary combinations of monomers using amide coupling, as these are easy to make and can rapidly generate a large library of compounds. The company now has a more diverse library of 70,000 compounds presented on microarrays. These can be screened against a protein target in a day, requiring just 5 mg of protein.

This microarray platform generates a relatively high number of hits, but many of them will be for compounds with similar structures, because the screen

HOW SMALL SHOULD YOU GO?

Miniaturization has been one of the triumphs in screening technology over the past decade or so, mainly because of advances in the automation of liquid handling, control software and detection systems. A few examples of ultra-high-throughput screening already exist, with Vertex in San Diego, California, for instance, performing the majority of its assays using 3,456-well microplates, in which each assay is done in a volume of just 1 µl.

Affymax, a drug-discovery firm based in Palo Alto, California, is reported to be working on a 20,000-well plate in which each well would have a volume of just 25 nl. And although most of the endeavour has been directed towards miniaturizing microplates, other ultra-high-throughput formats are being developed, the screening of microbead-attached combinatorial libraries, for instance, by companies such as Luminex in Austin, Texas, and Illumina in San Diego, California.

Although these examples are undoubtedly a taste of the future, miniaturization is not for everyone, and does not suit every purpose. Except when compound or protein amounts are critical factors, miniaturization is unlikely to make a big difference to the efficiency of primary screening, although doing assays in less time makes it easier to keep conditions standardized.

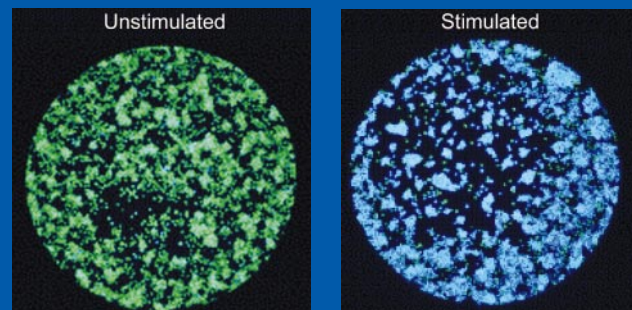
For Vertex’s cell-based assays, the transition from 384- to 3,456-well plates meant that the number of cells needed dropped from 10,000–40,000 per well to about 200. This means that the

company can work with cell types that are relatively difficult to get hold of, such as disease cells, says Paul Negulescu (below), vice-president of discovery biology at Vertex. In principle, 3,456-well plates could be used to study single cells, but at that level the cells’ ‘individuality’ starts to become a problem, giving uneven responses and so degrading the data.

A.S.



Vertex’s 3,456-well Nanowell plates enable ultra-high throughput.



picks up the activities of the monomer building blocks as well as the binary combinations.

High-content screening

The amount of information that can be gleaned from a screen can be increased by using cell-based systems. Screens such as those offered by Amersham Biosciences, Evotec OAI in Hamburg, Germany, and Vertex Pharmaceuticals in San Diego, California, allow complex biological data on lead-compound behaviour to be collected.

"Although the industry has been doing *in vitro* assays for a long time, there is a big increase in complexity when you start thinking about using whole cells," says John Anson, vice-president of systems development at Amersham. For instance, instead of just measuring the binding of a ligand to a receptor *in vitro*, you might now need to track the movement of a labelled molecule from the cytoplasm to the nucleus. Researchers are also beginning to measure more than one event at a time, for instance by using two different reporter molecules, and this is adding to the complexity.

The increased intricacy of assay systems is changing perceptions of the screening process. "The ability to track the internalization or translocation of a cellular component allows you to think more deeply about what you want to get out of a screen," says Paul Negulescu, vice-

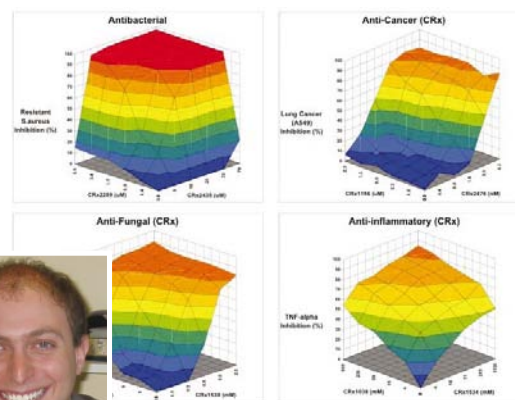
president of discovery biology at Vertex.

Although most researchers would admit that a degree of serendipity operates in screening for hits and leads, most screens are hypothesis-driven, using assays designed to test the effects of compounds on a particular protein target. But CombinatoRx, a two-year-old company based in Cambridge, Massachusetts, has taken a very different approach. It screens binary combinations of existing drugs to see whether drugs that have known effects when acting singly might have different, unexpected, effects when used in combination.

Double value

Most drugs do not, in fact, target single proteins, explains the company's chief executive, Alexis Borisov. Instead they interact with a number of targets at a variety of potencies. "Recognizing the inherent complexity of biological systems, we want drugs that will interact with multiple points in a pathway, rather than the 'sledgehammer' strategy of affecting just one key protein," he says, reversing the usual mantra that drugs should be as selective as possible.

The data generated by CombinatoRx's screens are built into 'interaction spaces' to illustrate the dose-response relationship of the two drugs in combination. At present, the company has



Alex Borisov: CombinatoRx's binary screens pick up complexity.

COMBINATORX

a screening library of 12.5 million binary combinations. And because all these molecules have already been approved by the US Food and Drug Administration, and are mostly off-patent, it should be possible rapidly to develop any hits for further testing. CombinatoRx plans to start clinical trials on its first sets of binary combinations later this year.

For all these new approaches to screening, the number of new compounds entering clinical trials in the coming years will be the ultimate measure of their success.

Adam Smith is editor of *Nature Reviews Drug Discovery*.

FRAGMENTING THE PROBLEM

Bioassay-based screening approaches search for hits with, for example, inhibitor constants at least in the low micromolar range. To get this much potency, a minimum number of interaction points between the molecule and the target protein are needed, which means that only larger compounds generate an initial hit.

Over the years, the compounds held by drug-discovery companies in their collections have been getting bigger, as screeners and medicinal chemists have chased the goal of potency. But this brings its own problems. "It is generally accepted that

larger compounds lead to more late-stage attrition," explains Harren Jhoti, co-founder and chief scientific officer of Astex Technology, a lead-discovery company based in Cambridge, UK. "Chemically refining large initial hits to work out which groups are important or not can be very time consuming." The highly sensitive screening techniques that are now emerging, such as nuclear

magnetic resonance and surface plasmon resonance, are providing the opportunity to search for lower-affinity hits, in the high micromolar or even low millimolar range. This opens up the possibility of screening for smaller starting compounds — 'chemical fragments' — which can then be built up into drug-like molecules by adding optimal functionalities, ideally using the structure of the target protein derived from X-ray crystallographic data. This, argues Jhoti, should allow for "a more directed path from hit to the lead than the hit-and-miss process of refining larger molecules". Astex now has libraries of chemical fragments that bind in the low millimolar range, and already has examples of nanomolar lead compounds that were generated from millimolar hits.

Another company working with fragments is Graffinity Pharmaceuticals, in Heidelberg, Germany, which is taking advantage of the sensitivity of its optically based screening platform to test microarrays of chemical fragments. Technological challenges aside, another barrier to the development of fragment-based approaches is the generally held perception that high potency in binding to a target is a prerequisite for further development of a compound. "There is a change of mindset needed in order to convince a chemist that a millimolar hit is going to be of use," says Jhoti.

A.S.

ASTEX TECHNOLOGY



Astex is building leads with X-ray crystallography.

Company	Products/activity	Location	URL
Instrumentation			
Acumen Bioscience	Fluorescence detectors for high- and moderate-throughput cell-based and cell-free multiplexed assays	Royston, UK	www.acumenbioscience.com
Agilent Technologies	Instruments and software for liquid chromatography (LC) and capillary LC, LC/MSD trap, AP-MALDI source, UV-visible spectroscopy	Palo Alto, California	www.agilent.com
Allegro Technologies	Pipetting and bulk-dispensing systems for nanolitre to microlitre quantities	Dublin, Ireland	www.allegro-technologies.com
Analytikjena	Instrumentation for molecular spectroscopy, affinity measurements	Jena, Germany	www.analytikjena.com
Applied Biosystems	Instruments for mass spectroscopy, fluorometric assays, functional protein-protein interactions	Foster City, California	www.appliedbiosystems.com
Asper Biotech	Microarray and genotyping hardware, software and services	Tartu, Estonia	www.asperbio.com
ASYS Hitech	Multichannel dispensers	Eugendorf, Austria	www.asyshitech.com
Berthold Technologies	Microplate readers	Bad Wildbad, Germany	www.bertholdtech.com
Biacore	Biacore S51 and Biacore 3000, instrumentation and evaluation software for the study of biomolecular interactions using surface plasmon resonance	Uppsala, Sweden	www.biacore.com
BMG Labtechnologies	Microplate readers	Offenburg, Germany	www.bmglabtech.com
Bruker	NMR and EPR spectrometers and related instrumentation	Billerica, Massachusetts	www.bruker.com
Bruker Daltonik	Automated mass spectrometry systems, proteomics hardware and software	Bremen, Germany	www.bdal.de
Caliper Technologies Corporation	LabChip microfluidic lab-on-a-chip systems	Mountain View, California	www.calipertech.com
CLONDIAG Chip Technologies	Array technology and microarray laboratory information management systems	Jena, Germany	www.clondiag.com
CyBio	Sample preparation, screening platforms, flash and glow luminescence detectors, incubators	Jena, Germany	www.cybio-ag.com
Genevac	Solvent removal systems for drying microplates	Ipswich, UK	www.genevac.com
Kendro Laboratory Products	Automated incubators and storage systems	Asheville, North Carolina	www.kendro.com
Luminex	Instrumentation for microsphere-based analysis of nucleic acids, antigen-antibody binding, enzymes and receptor-ligand interactions	Austin, Texas	www.luminexcorp.com
Multi Channel Systems	Automated injection and two-electrode voltage-clamp for <i>Xenopus</i> oocytes, multiple extracellular electrode recording and analysis systems	Reutlingen, Germany	www.multichannelsystems.com
PerkinElmer Analytical Instruments	UV/Visible, fluorescence, infrared spectrometers	Shelton, Connecticut	instruments.perkinelmer.com
Proteodyne Europe	BioCube systems for gene identification, analysis and high-throughput screening	Martinsried, Germany	www.proteodyne.com
SEPIAtec	HPLC/SPE equipment for high-throughput purification and fractionation of mixtures from natural products and combinatorial libraries	Berlin, Germany	www.sepiatec.com
Sirius	Instrumentation and analysis software for pKa, pH and membrane permeability measurement	Forest Row, UK	www.sirius-analytical.com
Tecan Group	High- and moderate-throughput screening workstations, assay development systems, protein fractionation, automated cell-culture systems	Maennedorf, Switzerland	www.tecan.com
The Automation Partnership	Robotic systems for large-scale industrial automation	Royston, UK	www.automationpartnership.com
Thermo Finnigan	Mass spectrometry and chromatography systems and services	San Jose, California	www.thermofinnigan.com
Thermo Labsystems	Pipettes and tips, microplates and readers, magnetic-particle processors, research reagents and diagnostic kits	Vantaa, Finland	www.thermolabsystems.fi
Zymark Corporation	Assay automation, liquid handling, ultra-high-throughput screening	Hopkinton, Massachusetts	www.zymark.com
Reagents			
BD Biosciences	Products for cell and tissue culture, proteomics, oxygen biosensor systems	Bedford, Massachusetts	www.bdbiosciences.com
Corning	96- and 384-well coated microplates	Acton, Massachusetts	www.corning.com/lifesciences
Eppendorf	Reagents and instruments for high-throughput PCR	Hamburg, Germany	www.eppendorf.com
IBA	ProteoRapid <i>Strep</i> -tag <i>in vitro</i> transcription/translation system	Göttingen, Germany	www.iba-go.de
Mirus	TransIT transfection reagents	Madison, Wisconsin	www.genetransfer.com
PanVera	Assays for potential drug target proteins	Madison, Wisconsin	www.panvera.com
Perbio	Reagents for protein chemistry, proteomics and high-throughput screening	Helsingborg, Sweden	www.perbio.com
PerkinElmer Life Sciences	High-throughput screening, imaging, liquid handling, readers, labelling reagents	Boston, Massachusetts	lifesciences.perkinelmer.com
PicoRapid Technologie	Activated PicoSlides for microarray spotting, customized PicoArrays, non-contactspotting, microarray services	Bremen, Germany	www.picorapid.de
Pierce Biotechnology	SearchLight cytokine arrays, kinase assay platform, coated microplates	Rockford, Illinois	www.piercenet.com
QIAGEN	LiquiChip bead-based assay system, Ni-NTA magnetic agarose beads, Ni-NTA HisSorb strips and plates	Hilden, Germany	www.qiagen.com
USB	Enzymes and reagents for manual DNA sequencing and molecular biology	Cleveland, Ohio	www.usbweb.com
Vysis	Genomic DNA arrays, reader, software and reagents	Downers Grove, Illinois	www.vysis.com
Software			
Accelrys	Software for management of compounds and biological data in high-throughput screening and plate-based tests, database query and reporting software	San Diego, California	www.accelrys.com
Biomax Informatics	Customized bioinformatics for biological data management and analysis	Martinsried, Germany	www.biomax.com
Cimarron Software, Inc.	Laboratory workflow and analysis software and services	Salt Lake City, Utah	www.cimssoft.com
GeneData	Software for analysis of high-throughput screening data, data quality control, <i>in silico</i> lead candidate identification, screening	Basel, Switzerland	www.genedata.com
IBM	Information storage, management and analysis software and services	White Plains, New York	www-3.ibm.com/solutions/lifesciences
InformMax	Software for genomics, proteomics and gene-expression data	Bethesda, Maryland	www.informaxinc.com
LION Bioscience	Software for genomics and microarray data analysis, database searching	Heidelberg, Germany	www.lionbioscience.com
NuGenesis	Scientific data management system	Westborough, Massachusetts	www.nugeneis.com
PubGene	Bioinformatics analysis tool for genomics and proteomics	Oslo, Norway	www.pubgene.com

Science Factory	Enzymology database, bioinformatics platform for sequence, expression and structural data	Cologne, Germany	www.science-factory.com
Spotfire	Analytical and statistical software for drug discovery	Somerville, Massachusetts	www.spotfire.com
SPSS	Statistical and data-mining software	Chicago, Illinois	www.spss.com
Tripos	Drug discovery software, LeadQuest compound library, software consulting	St Louis, Missouri	www.tripos.com

Services

Affibody	Affibody binding proteins	Bromma, Sweden	www.affibody.com
Affitech	Human antibody discovery <i>in vitro</i> from libraries and patients	Oslo, Norway	www.affitech.com
Atugen	Discovery and validation of <i>in vitro</i> and <i>in vivo</i> therapeutic targets	Berlin, Germany	www.atugen.com
BioChip Technologies	Pharm-O-Kin Screen customized genotyping service	Freiburg im Breisgau, Germany	www.genescan.com
BioFocus	Compound design and synthesis, molecular modelling, quantitative structure–activity relationships, assay development, high-throughput screening	Sittingbourne, UK	www.biofocus.com
Chemical Diversity Labs	Molecular libraries for 'hit' identification and 'hit-to-lead' structure–activity relationships, lead optimization and scale-up	San Diego, California	www.chemdiv.com
Cytomx	Gel analysis, mass spectroscopy and amino-terminal sequencing, recombinant protein expression, expression profiling, cell-line production	Cambridge, UK	www.cytomx.com
EUGENEX Biotechnologies	Development of test cell lines	Taegervilen, Switzerland	www.eugenex.com
Evotec OAI	Compound libraries, assay development, high-throughput screening, lead optimization and medicinal chemistry	Hamburg, Germany	www.evotecai.com
Graffinity Pharmaceuticals	Chemical microarrays and label-free imaging of protein–ligand interactions	Heidelberg, Germany	www.graffinity.com
Illumina	Oligonucleotide synthesis, automated large-scale genotyping systems based on microbead technology	San Diego, California	www.illumina.com
Invitrogen	Molecular biology products	Carlsbad, California	www.invitrogen.com
Jerini Array Technologies	Peptide- and protein-domain microarray technology, substrate identification service for kinases	Berlin, Germany	www.jerini.com/array
KeyNeurotek	Functional screening for target identification, validation and drug testing	Magdeburg, Germany	www.keyneurotek.de
Kiadis	Complex mixture screening using label-free or label-based formats	Leiden, The Netherlands	www.kiadis.com
WITA Proteomics	Predictive toxicology; evaluation of lead compounds	Berlin, Germany	www.wita-proteomics.com

General

3D Pharmaceuticals	In-house drug discovery	Yardley, Pennsylvania	www.3dp.com
Affymax	Drug discovery	Palo Alto, California	www.affymax.com
Amgen	Protein and small-molecule drug discovery and development	Thousand Oaks, California	www.amgen.com
Apogent Technologies	Equipment and products for cell and molecular biology	Portsmouth, New Hampshire	www.apogent.com
Argenta Discovery	High-throughput screening, sample management, informatics	Harlow, UK	www.argentadiscovery.com
Astex Technology	Computational screening of virtual libraries, high-throughput X-ray crystallography and software for protein–ligand structure determination	Cambridge, UK	www.astex-technology.com
Amersham Biosciences	Scintillation proximity assay beads and kits, LEADseeker beads and imaging system, reagents and imaging systems for a range of assays	Piscataway, New Jersey	www.amershambiosciences.com
CombinatoRx	Lead compound identification and screening	Boston, Massachusetts	www.combinatorx.com
Cytos Biotechnology	DELphi high-throughput functional genomics technology	Zurich, Switzerland	www.cytos.com
De Novo Pharmaceuticals	Screening of virtual libraries, in-house drug discovery	Cambridge, UK	www.denovopharma.com
Genovar diagnostics	DNA and RNA extraction, genotype analysis of drug-metabolizing enzymes and custom genotyping services for polymorphic genes	Sittingbourne, UK	www.genovar.co.uk
Jurilab	DNA gene bank, mutation searching	Kuopio, Finland	www.jurilab.com
Laxdale	Drug development for psychiatric illnesses	Stirling, UK	www.laxdale.co.uk
Merck	Drug discovery, development and production	Whitehouse Station, New Jersey	www.merck.com
Millipore	Multiscreen sample preparation and assays, compound synthesis, cell-based and receptor assays	Bedford, Massachusetts	millipore.com
MWG Biotech	Design, production and automation of high-throughput sequencing and microarray systems	Ebersberg, Germany	www.mwg-biotech.com
NIMBUS Biotechnology	TRANSIL lipid membranes, screening for membrane affinity/lipophilicity, screening system for transmembrane proteins	Leipzig, Germany	www.nimbus-biotech.com
Roche Applied Science	PCR systems, translation systems, microtitre plates, transfection reagents, custom protein expression service	Indianapolis, Indiana	www.roche-applied-science.com
SuNyx Surface Nanotechnologies	Development of nanofluidic substrates for detection and identification of biomolecules using MALDI mass spectroscopy and fluorescence detection	Cologne, Germany	www.sunyx.de
The Genetics Company	Identification of molecular targets, diagnostic markers, and potential active compounds from DNA sequence and genetic information	Zürich, Switzerland	www.the-genetics.com
Vertex	Small-molecule drug discovery	Cambridge, Massachusetts	www.vpharm.com
Xantos Biomedicine	Target screening of DNA libraries and related services	Martinsried, Germany	www.xantos.de

● See advertisement

Correction: In the Table of suppliers in the feature "DNA microarray technology" (*Nature* **416**, 884–895; 2002), the Product/activity entries for Memorec Stoffel and Schleicher & Schuell were inadvertently transposed and should read

Memorec Stoffel	PIQOR technology for producing custom and generic murine and human cDNA arrays	Cologne, Germany	www.memorec.com
Schleicher & Schuell	Coated slides for microarrays; nylon membranes for PCR-based arrays	Dassel Germany	www.s-und-s.de

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Working with the Framework

Can social engineering work in science? The European Commission (EC) last month took steps to answer this question as it advanced its sixth Framework — a four-year, 17.5-billion-euro (US\$17.6-billion) plan to distribute funds based in part on researchers' abilities to form pan-European research networks.

The initial signs appear positive. In March, the EC put out a call for research ideas in several broad categories. Those ideas are to be evaluated both on their scientific merit as well as on the number of institutions listed. Last month the EC reported that it had received 15,000 proposals. But some scientists who have submitted proposals say that number may be a bit misleading, as some proposals list 50–60 collaborators and some groups appear on several separate proposals.

These numbers can be interpreted in two ways. Looking at the sheer volume of responses, one could say that the EC accomplished its goal of getting scientists to find out who is doing similar or complementary work in other parts of Europe. But examining the redundancy, one could also say that the plan has led to a duplication in proposals, as scientists strive to increase their odds of being a part of a group that makes its way through the funding process.

As a survey of interest in pan-European research projects, the Framework is a qualified success. But as a mechanism to make researchers who have never worked together cooperate, the result is likely to be less positive. Even if a fraction of the respondents submit formal proposals by the end of the year, it is likely that only a few of those will be funded. And of those, it is unclear how they will be managed and evaluated. Coaxing groups to work together seems to be an admirable goal — but making it happen effectively remains difficult to accomplish.

Paul Smaglik

Naturejobs editor



Contents

MOVERS

Fresh vistas for discoverer of Lyme disease; split job in Taiwanese astronomy; Sloan-Kettering gets computational; and more

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Transitions

X Mitchell Kronenberg is to replace **Howard Grey** as president and scientific director of the La Jolla Institute for Allergy and Immunology when Grey retires in September 2003. Kronenberg joined the institute in 1997 and is currently head of developmental immunology there.

X This month saw **Steven Teitelbaum** become president of the Federation of American Societies for Experimental Biology. Teitelbaum is a professor of pathology at the Washington University School of Medicine in St Louis.

X Robert Booth is to be senior vice-president of research and development at Celera Genomics in Rockville, Maryland. Booth comes to the company from Hoffmann-La Roche, where he was senior vice-president of the inflammatory- and viral-diseases business unit.

X Donal Geaney has resigned as chairman and chief executive of Elan in Dublin, and **Thomas Lynch** has resigned as the company's vice-chairman.

X Ian McDonald has been named vice-president of drug discovery at Structural Genomics in San Diego. Previously, he was vice-president of drug discovery at Structural Bioinformatics.

X Brian Barber left Aventis Pasteur in Toronto to join Mojave Therapeutics in Hawthorne, New York, as chief scientific officer.

X Gary Woodnutt is to join Diversa, a biotech company in San Diego, as senior vice-president, pharmaceutical development. His previous position was vice-president of microbiology at GlaxoSmithKline.

MEDICINE

This month saw **Allen Steere** switch both hospitals and universities. Steere, who is probably best known as the discoverer of Lyme disease, was chief of rheumatology and immunology at the New England Medical Center in Boston. He is now director of the clinical rheumatology and allergy programme at Massachusetts General Hospital also in Boston, and will run a lab with 8–12 researchers. At the same time, his teaching affiliation changes from professor at Tufts School of Medicine to professor at Harvard Medical School.

PROTEOMICS

As a result of corporate restructuring, **Leigh Anderson**, co-founder of proteomics company Large Scale Biology in Vacaville, California, has stepped down as one of the firm's directors, although he will continue to serve as a consultant for the company. The company merged with Biosource Technologies in 1999. Over the next few months, Anderson is planning to assess the field of proteomics. He acknowledges that the field has drawn its share of hype and relabelling, but he thinks that it will eventually have a clinical impact — especially in diagnostics. “The question for me is how that discipline will get translated into tools and results that will have a significant near-term medical impact,” Anderson says.

BIOINFORMATICS

Chris Sander is set to head the new Computational Biology Center at the Memorial Sloan-Kettering Cancer Center in New York. He will direct a programme of biological research using computational techniques and forge alliances between computational biologists and cancer researchers.

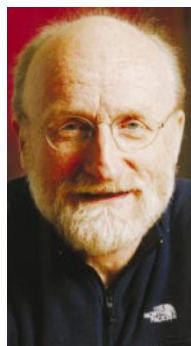
Sander is the editor of the journal *Bioinformatics* and he advises the US National Institutes of Health, the Mayo Clinic Genomic Center and the IBM Deep Computing Initiative. He previously worked as chief information science officer with Millennium Pharmaceuticals and at the European Bioinformatics Institute. The new centre will be a key interface for the research collaborations between Sloan-Kettering, Cornell University and Rockefeller University.

MATERIALS

Angela Belcher, a young, high-profile, material-sciences researcher, will leave the University of Texas at Austin for the Massachusetts Institute of Technology (MIT) this autumn. Belcher, whose work includes using viruses to grow synthetic



Leigh Anderson



Chris Sander

materials, was attracted to MIT in part by its new nanotechnology lab and the chance to interact with the area's biotechnology community. “It's going to be a very exciting place for nanoscience and bioengineering,” Belcher says. Her graduate students and postdocs seem to agree — most have decided to join her when the lab opens in October. Until then, she will commute between the two campuses.

PHARMACEUTICALS

Biotechnology company Eyetech Pharmaceuticals this month welcomed **Anthony Adamis** from Harvard Medical School as its new senior vice-president of research and chief scientific officer. Eyetech specializes in the development and commercialization of drugs for treating eye diseases.

Adamis, who has been affiliated with Eyetech since its inception in 2000, was attracted to the company because it has drugs in the clinical pipeline that target vascular endothelial growth factor. “That's an area I've worked in for 10 years,” he says.

He will establish the Eyetech Research Laboratory in Boston, even though the company is based in New York. But he will still divide his research between basic and applied work, as the company needs to understand the pathogenesis of the eye diseases it intends to treat. He expects to have more resources than he would if he had stayed in an academic lab. “I'll be able to do science on a scale I haven't been able to do as an independent investigator,” he says.

ASTRONOMY

Harvard University astronomer **Paul Ho** has been splitting his time between the United States and Taiwan ever since he took over as acting director of Taiwan's Institute of Astronomy and Astrophysics in Taipei. He stepped into the breach last month when the previous director, **Fred Lo**, left to head the US National Radio Astronomy Observatory (see *Nature* **417**, 781; 2002).

This is a hectic time for Taiwanese astronomy, as the country is involved in three important projects that are scheduled to come online this year — the Submillimeter-Wave Array, a set of antennas on Mauna Kea in Hawaii; a small interferometer to study microwave background radiation; and an experiment to look at Kuiper-belt objects.

“My purpose here is to stabilize things and make sure that the instrumentation goes forward,” Ho says. But he says he is uncertain how long he will remain in the position, as the search is under way for a permanent director.

CONTACTING US AT MOVERS

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